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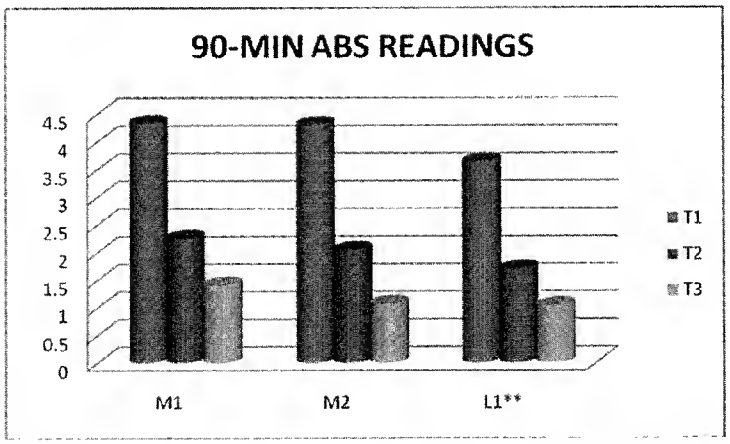

Figure 3

(57) Abstract: The present invention provides novel compositions to transfect cells for production of growth hormone (GH). These novel compositions also are used to produce germline transgenic birds that can successfully pass the transgene encoding growth hormone to their offspring. These novel compositions include components of vectors such as a vector backbone, a novel promoter, and a gene of interest that encodes for GH, and the vectors comprising these components. In one embodiment these vectors are transposon-based vectors. The present invention also provides methods of making these compositions and methods of using these compositions for the production of GH in vitro and in vivo. In one embodiment the GH is human (h)GH.

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NOVEL VECTORS FOR PRODUCTION OF GROWTH HORMONE

FIELD OF THE INVENTION

The present disclosure relates to compositions and methods for the production of growth hormone (GH). In particular, the disclosure relates to transposon based vectors and their use in methods for the efficient expression of GH.

BACKGROUND OF THE INVENTION

Manufacture of therapeutic proteins, such as GH, is an expensive process. Companies using recombinant techniques to manufacture GH are working at capacity and usually have a long waiting list to access their fermentation facilities. What is needed is a new, efficient, and economical approach to make GH *in vitro* or *in vivo*.

SUMMARY

The present invention addresses these needs by providing novel compositions which can be used for production of GH. These novel compositions include components of vectors such as vector backbones (SEQ ID NOs:1-13), novel promoters (SEQ ID NOs:14-15), and a gene of interest that encodes for GH. The present vectors comprise an insulator element located between the transposon insertion sequences and the multicloning site on the vector. In one embodiment, the insulator element is selected from the group consisting of an HS4 element, a lysozyme replicator element, a combination of a lysozyme replicator element and an HS4 element, and a matrix attachment region element. The vectors comprising each of these vector components are shown in SEQ ID NOs:17-39. In one embodiment these vectors are transposon-based vectors. The present invention also provides methods of making these compositions and methods of using these compositions for the production of GH *in vitro* or *in vivo*. In one embodiment the GH is human (h)GH. These vectors have been used to transfect germline cells of birds through cardiac injection. The transgene in these vectors has been successfully passed through two generations of offspring, demonstrating stable integration and inheritance of the transgene.

Prokaryotic cells or eukaryotic cells may be transfected. It is to be understood that different cells may be transfected *in vitro* or *in vivo* with one of the presently disclosed compositions, provided the cells contain protein synthetic biochemical pathways for the expression of GH. For example, both prokaryotic cells and eukaryotic cells may be transfected with one of the disclosed compositions. In certain embodiments, animal or plant cells are transfected. Animal cells are preferred cells and include, for example, mammalian cells and

avian cells. Animal cells that may be transfected include, but are not limited to, Chinese hamster ovary (CHO) cells, CHO-K1 cells, chicken embryonic fibroblasts, HeLa cells, Vero cells, FAO (liver cells), human 3T3 cells, A20 cells, EL4 cells, HepG2 cells, J744A cells, Jurkat cells, P388D1 cells, RC-4B/c cells, SK-N-SH cells, Sp2/mIL-6 cells, SW480 cells, 3T6 Swiss cells, human ARPT-19 (human pigmented retinal epithelial) cells, LMH cells, LMH2a cells, tubular gland cells, or hybridomas. Avian cells include, but are not limited to, LMH, LMH2a cells, chicken embryonic fibroblasts, and tubular gland cells.

In one embodiment, avian cells are transfected with one of the disclosed compositions. In a specific embodiment, avian hepatocytes, hepatocyte-related cells, or tubular gland cells are transfected. In certain embodiments, chicken cells are transfected with one of the disclosed compositions. In one embodiment, chicken tubular gland cells, chicken embryonic fibroblasts, chicken LMH2A or chicken LMH cells are transfected with one of the disclosed compositions. Chicken LMH and LMH2A cells are chicken hepatoma cell lines; LMH2A cells have been transformed to express estrogen receptors on their cell surface.

In other embodiments, mammalian cells are transfected with one of the disclosed compositions. In one embodiment, Chinese hamster ovary (CHO) cells, ARPT-19 cells, HeLa cells, Vero cells, FAO (liver cells), human 3T3 cells, or hybridomas are transfected for GH production. In a specific embodiment, CHO-K1 cells or ARPT-19 cells are transfected with one of the disclosed compositions.

The present invention provides compositions and methods for efficient production of GH, particularly hGH, *in vitro* or *in vivo*. This method enables production of large quantities of GH *in vitro*. Large quantities of GH include amounts in the gram and kilogram range, depending on the capacity of the culture method employed. These vectors may also be used *in vivo* to transfect germline cells in birds which can be bred and pass the transgene through several generations. These vectors also may be used for the production of GH *in vivo*, for example, for deposition in an egg.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents a schematic representation of the construction of SEQ ID NO:14.

Figure 2 presents a schematic representation of the construction of SEQ ID NO:15.

Figure 3 presents GH ELISA results. T1, T2, and T3 are displayed in order from left to right at each of M1, M2, and L1.

Figure 4 presents GH ELISA results (absorbance readings). T1, T2, T3, T4, T5, and T6 are displayed in order from left to right at each of M1, M2, M3, and L1.

Figure 5 presents estimated protein concentration results. T1, T2, T3, T4, T5, and T6 are displayed in order from left to right at each of M1, M2, M3, and M4.

Figure 6 presents GH ELISA results. 171 (SEQ ID NO:27), Hybrid v1, Hybrid v2, and Hybrid v3 are displayed in order from left to right at each of M1, M2, M3, and M4. See

Figure 7 presents GH ELISA results for Experiment 1136. C1, T1, T2, and T3 are displayed in order from left to right at each of M1, M2, M3, M4, and L1.

Figure 8 presents additional GH ELISA results for Experiment 1136. C1, T4, T5, T6, T7, and T8 are displayed in order from left to right at each of M1, M2, M3, M4, and L1.

Figure 9 presents additional GH ELISA results for Experiment 1136. C1, T1, T2, T3, T4, T5, T6, T7, and T8 are displayed in order from left to right at each of M1, M2, M3, M4, and L1.

Figure 10 presents GH ELISA results for Experiment 1151. T1 and T2 are displayed in order from left to right at each of M1 and M2

Figure 11 is a schematic representation of MAR elements incorporated into the transposon backbone. The MAR elements flanked the multiple cloning site which is where the gene of interest and its regulatory components were inserted. The MAR elements can be replaced with the HS4 or LysRep elements, or combinations thereof.

Figure 12 is a schematic representation of 3xFlag-hGH output from LMH2A cells (A1A clone) in a perfusion system. EC and Harvest are displayed in order from left to right at each of M1, M2, M3, M4, M5, and M6.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

The present invention provides novel vectors and vector components for use in GH production. In one embodiment, the vectors are used for transfecting cells for GH production *in vitro*. In another embodiment, the vectors are used for transfecting cells for GH production *in vivo*. The present invention also provides methods to make these vector components, the vectors themselves, and methods for using these vectors to transfect cells so these cells produce GH *in vitro*. Any cell with protein synthetic capacity may be used. It is to be understood that different cells may be transfected *in vitro* or *in vivo* with one of the presently disclosed compositions, provided the cells contain protein synthetic biochemical pathways for the expression of the GH gene. For example, both prokaryotic cells and eukaryotic cells may be transfected with one of the disclosed compositions. In certain embodiments, animal or plant cells are transfected. Animal cells are preferred cells and include, for example, mammalian cells and avian cells. Cells that may be transfected include, but are not limited to, Chinese hamster ovary (CHO) cells, chicken embryonic fibroblasts, HeLa cells, Vero cells, FAO cells (liver cells), human 3T3 cells, A20 cells, EL4 cells, HepG2 cells, J744A cells, Jurkat cells, P388D1 cells, RC-4B/c cells, SK-N-SH

cells, Sp2/mIL-6 cells, SW480 cells, 3T6 Swiss cells, CHO-K1 cells, ARPE-19 cells, LMH cells, LMH2a cells, tubular gland cells, or hybridomas. Avian cells include, but are not limited to, LMH, LMH2a cells, chicken embryonic fibroblasts, and tubular gland cells. In one embodiment, avian cells are transfected with one of the disclosed compositions. In a specific embodiment, avian hepatocytes, hepatocyte-related cells, or tubular gland cells are transfected. In certain embodiments, chicken cells are transfected with one of the disclosed compositions. In one embodiment, chicken tubular gland cells, chicken embryonic fibroblasts, chicken LMH2A or chicken LMH cells are transfected with one of the disclosed compositions. Chicken LMH and LMH2A cells are chicken hepatoma cell lines; LMH2A cells have been transformed to express estrogen receptors on their cell surface.

In other embodimcnts, mammalian cells are transfectcd with one of the disclosed compositions. In one embodiment, CHO cells, HeLa cells, Vero cells, FAO cells (liver cells), human 3T3 cells, or hybridomas are transfected.

These vectors may also be used *in vivo* to produce transgenic animals by transfecting germline cells in animals such as birds which can be bred and then which pass the transgene encoding GH through several generations. These vectors also may be used *in vivo* to transform cells such as secretory cells to produce GH, for example, for deposition into an egg of the bird.

In one embodiment, the vectors of the present invention contain a gene encoding for GH production, particularly hGH production, by transfected cells. As used herein, the term growth hormone refers to GH protein that is encoded by a gene that is either a naturally occurring or a codon-optimized gene. As used herein, the term "codon-optimized" means that the DNA sequence has been changed such that where several different codons code for the same amino acid residue, the sequence selected for the gene is the one that is most often utilized by the cell in which the gene is being expressed. For example, in some embodiments, the gene of interest is expressed in LMH or LMH2A cells and includes codon sequences that are preferred in that cell type.

A. Vectors & Vector Components

The following paragraphs describe the novel vector components and vectors employed in the present invention.

1. *Backbone Vectors*

The backbone vectors provide the vector components minus the gene of interest (GOI) that encodes GH. In one embodiment, transposon-based vectors are used. The present vectors further comprise an insulator element located between the transposon insertion sequences and the multicloning site on the vector. In one embodiment, the insulator element is selected from the

group consisting of an HS4 element, a lysozyme replicator element, a combination of a lysozyme replicator element and an HS4 element, and a matrix attachment region element.

a. *Transposon-Based Vector Tn-MCS #5001 (p5001) (SEQ ID NO:1)*

Linear sequences were amplified using plasmid DNA from pBluescriptII sk(-) (Stratagene, La Jolla, CA), pGWIZ (Gene Therapy Systems, San Diego, CA), pNK2859 (Dr. Nancy Kleckner, Department of Biochemistry and Molecular Biology, Harvard University), and synthetic linear DNA constructed from specifically designed DNA Oligonucleotides (Integrated DNA Technologies, Coralville, IA). PCR was set up using the above referenced DNA as template, electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. DNA bands corresponding to the expected size were excised from the gel and purified from the agarose using Zymo Research's Clean Gel Recovery Kit (Orange, CA). The resulting products were cloned into the Invitrogen's PCR Blunt II Topo plasmid (Carlsbad, CA) according to the manufacturer's protocol.

After sequence verification, subsequent clones were selected and digested from the PCR Blunt II Topo Vector (Invitrogen Life Technologies, Carlsbad, CA) with corresponding enzymes (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. The linear pieces were ligated together using Stratagene's T4 Ligase Kit (La Jolla, CA) according to the manufacturer's protocol. Ligated products were transformed into *E. coli* Top10 cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to the manufacturer's protocol. Transformed bacterial cells were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) for 1 hour at 37°C then spread to LB (Luria-Bertani) agar plates supplemented with 100 μg/ml ampicillin (LB/amp plates). These plates were incubated overnight at 37°C. Resulting colonies were picked into LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on a U.V. transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in a minimum of 250 ml of LB/amp broth. Plasmid DNA was harvested using Qiagen's Maxi-Prep Kit according to the manufacturer's protocol (Chatsworth, CA). The DNA was used as a sequencing template to verify that the pieces were ligated together accurately to form the desired vector sequence. All sequencing was performed using Beckman Coulter's CEQ 8000 Genetic Analysis System. Once a clone was identified that consisted of the desired sequence, the DNA was isolated for use in cloning in specific genes of interest.

b. *Preparation of Transposon-Based Vector TnX-MCS #5005 (p5005)*

This vector (SEQ ID NO:2) is a modification of p5001 (SEQ ID NO:1) described above in section 1.a. The multiple cloning site (MCS) extension was designed to add unique restriction

sites to the MCS of the pTn-MCS vector (SEQ ID NO:1), creating pTnX-MCS (SEQ ID NO:2), in order to increase the ligation efficiency of constructed cassettes into the backbone vector. The first step was to create a list of all non-cutting enzymes for the current pTn-MCS DNA sequence (SEQ ID NO:1). A linear sequence was designed using the list of enzymes and compressing the restriction site sequences together. Necessary restriction site sequences for XhoI and PspOMI (New England Biolabs, Beverly, MA) were then added to each end of this sequence for use in splicing this MCS extension into the pTn-MCS backbone (SEQ ID NO:1). The resulting sequence of 108 bases is SEQ ID NO:16 shown in the Appendix. A subset of these bases within this 108 base pair sequence corresponds to bases 4917-5012 in SEQ ID NO:4 (discussed below).

For construction, the sequence was split at the NarI restriction site and divided into two sections. Both 5' forward and 3' reverse oligonucleotides (Integrated DNA Technologies, San Diego, CA) were synthesized for each of the two sections. The 5' and 3' oligonucleotides for each section were annealed together, and the resulting synthetic DNA sections were digested with NarI then subsequently ligated together to form the 108 bp MCS extension (SEQ ID NO:16). PCR was set up on the ligation, electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. DNA bands corresponding to the expected size were excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA). The resulting product was cloned into the PCR Blunt II Topo Vector (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol.

After sequence verification of the MCS extension sequence (SEQ ID NO:16), a clone was selected and digested from the PCR Blunt II Topo Vector (Invitrogen Life Technologies, Carlsbad, CA) with XhoI and PspoMI (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. The pTn-MCS vector (SEQ ID NO:1) also was digested with XhoI and PspOMI (New England Biolabs, Beverly, MA) according to the manufacturer's protocol, purified as described above, and the two pieces were ligated together using Stratagene's T4 Ligase Kit (La Jolla, CA) according to the manufacturer's protocol. Ligated product was transformed into *E. coli* Top10 cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according the manufacturer's protocol. Transformed bacterial cells were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) for 1 hour at 37°C then spread onto LB agar plates supplemented with 100 μg/ml ampicillin (LB/amp plates). All plates were incubated overnight at 37°C. Resulting colonies were picked into LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in a

minimum of 250 mls of LB/amp broth. Plasmid DNA was harvested using a Qiagen Maxi-Prep Kit (column purification) according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). The DNA was then used as a sequencing template to verify that the changes made in the vector were the desired changes and that no further changes or mutations occurred. All sequencing was performed using Beckman Coulter's CEQ 8000 Genetic Analysis System. Once a clone was identified that contained the multiple cloning site extension, the DNA was isolated and used for cloning specific genes of interest.

c. *Preparation of Transposon-Based Vector TnHS4FBV #5006 (p5006)*

This vector (SEQ ID NO:3) is a modification of p5005 (SEQ ID NO:2) described above in section 1.b. The modification includes insertion of the HS4 βeta globin insulator element on both the 5' and 3' ends of the multiple cloning site. The 1241 bp HS4 element was isolated from chicken genomic DNA and amplified through polymerase chain reaction (PCR) using conditions known to one skilled in the art. The PCR product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. DNA bands corresponding to the expected size of the HS4 βeta globin insulator element were excised from the agarose gel and purified using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA).

Purified HS4 DNA was digested with restriction enzymes NotI, XhoI, PspOMI, and MluI (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. The digested DNA was then purified using a Zymo DNA Clean and Concentrator kit (Orange, CA). To insert the 5' HS4 element into the MCS of the p5005 vector (SEQ ID NO:2), HS4 DNA and vector p5005 (SEQ ID NO:2) were digested with NotI and XhoI restriction enzymes, purified as described above, and ligated using Stratagene's T4 Ligase Kit (La Jolla, CA) according to the manufacturer's protocol. To insert the 3' HS4 element into the MCS of the p5005 vector (SEQ ID NO:2), HS4 and vector p5005 DNA (SEQ ID NO:2) were digested with PspOMI and MluI, purified, and ligated as described above. Ligated product was transformed into *E. coli* Top10 cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to the manufacturer's protocol. Transformed bacterial cells were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) for 1 hour at 37°C then spread onto LB agar plates supplemented with 100 μg/ml ampicillin (LB/amp plates). These plates were incubated overnight at 37°C. Resulting colonies were picked into LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in at least 250 mls of LB/amp broth and plasmid DNA was harvested using a Qiagen Maxi-Prep Kit according to the

manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). The DNA was then used as sequencing template to verify that any changes made in the vector were the desired changes and that no further changes or mutations occurred. All sequencing was performed using Beckman Coulter's CEQ 8000 Genetic Analysis System. Once a clone was identified that contained both HS4 elements, the DNA was isolated and used for cloning in specific genes of interest.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* bacteria containing the plasmid of interest were grown in 500 ml of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight in a shaking incubator. Plasmid DNA was isolated from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 µL of PCR-grade water and stored at -20°C until needed.

d. *Preparation of Transposon-Based Vector pTn10 HS4FBV #5012*

This vector (SEQ ID NO:4) is a modification of p5006 (SEQ ID NO:3) described above under section 1.c. The modification includes a base pair substitution in the transposase gene at base pair 1998 of p5006. The corrected transposase gene was amplified by PCR from template DNA, using PCR conditions known to one skilled in the art. PCR product of the corrected transposase was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. DNA bands corresponding to the expected size were excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA).

Purified transposase DNA was digested with restriction enzymes NruI and StuI (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Digested DNA was purified from restriction digests using a Zymo DNA Clean and Concentrator kit (Zymo Research). To insert the corrected transposase sequence into the MCS of the p5006 vector (SEQ ID NO:3), the transposase DNA and the p5006 vector (SEQ ID NO:3) were digested with NruI and StuI, purified as described above, and ligated using a Stratagene's T4 Ligase Kit (La Jolla, CA) according to the manufacturer's protocol. Ligated product was transformed into *E. coli* Top10 cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to the manufacturer's protocol. Transformed cells were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) for 1 hour at 37°C before spreading onto LB agar plates supplemented with 100 µg/ml ampicillin (LB/amp plates). All plates were incubated overnight at 37°C. Resulting colonies were picked into LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in at

least 250 ml of LB/amp broth. The plasmid DNA was harvested using a Qiagen Maxi-Prep Kit according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). The DNA was then used as a sequencing template to verify that the changes made in the vector were desired changes and that no further changes or mutations occurred. All sequencing was performed using a Beckman Coulter CEQ 8000 Genetic Analysis System. Once a clone was identified that contained the corrected transposase sequence, the DNA was isolated and used for cloning in specific genes of interest.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* bacteria containing the plasmid of interest was grown in 500 mL of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight in a shaking incubator. Plasmid DNA was isolated from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 µL of PCR-grade water and stored at -20°C until needed.

e. *Preparation of Transposon-Based Vector pTn-10 MARFBV #5018*

This vector (SEQ ID NO:5) is a modification of p5012 (SEQ ID NO:4) described above under section 1.d. The modification includes insertion of the chicken 5' Matrix Attachment Region (MAR) on both the 5' and 3' ends of the multiple cloning site. To accomplish this, the 1.7 kb MAR element was isolated from chicken genomic DNA and amplified by PCR. PCR product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. DNA bands corresponding to the expected size were excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA).

Purified MAR DNA was digested with restriction enzymes NotI, XhoI, PspOMI, and MluI (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Digested DNA was purified from agarose using a Zymo DNA Clean and Concentrator kit (Zymo Research, Orange CA). To insert the 5' MAR element into the MCS of p5012, the purified MAR DNA and p5012 were digested with Not I and Xho I, purified as described above, and ligated using Stratagene's T4 Ligase Kit (La Jolla, CA) according to the manufacturer's protocol. To insert the 3' MAR element into the MCS of p5012, the purified MAR DNA and p5012 were digested with PspOMI and MluI, purified, and ligated as described above. Ligated product was transformed into *E. coli* Top10 cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to the manufacturer's protocol. Transformed cells were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) for 1 hour at 37°C and then spread onto LB agar plates supplemented with 100 µg/ml ampicillin (LB/amp plates). All plates were incubated overnight at 37°C. Resulting colonies were picked into LB/amp broth for overnight growth at

37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in a minimum of 250 ml of LB/amp broth, and plasmid DNA was harvested using a Qiagen Maxi-Prep Kit according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). Column purified DNA was used as a sequencing template to verify that the changes made in the vector were the desired changes and that no further changes or mutations occurred. All sequencing was performed using a Beckman Coulter CEQ 8000 Genetic Analysis System. Once a clone was identified that contained both MAR elements, the DNA was isolated and used for cloning in specific genes of interest.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* bacteria containing the plasmid of interest were grown in 500 mL of LB broth (supplemented with an appropriate antibiotic) at 37°C in a shaking incubator. Plasmid DNA was isolated from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 µL of PCR-grade water and stored at -20°C until needed.

f. *Preparation of Transposon-Based Vector TnLysRep #5020*

The vector (SEQ ID NO:6) included the chicken lysozyme replicator (LysRep or LR2) insulator elements to prevent gene silencing. Each LysRep element was ligated 3' to the insertion sequences (IS) of the vector. To accomplish this ligation, a 930 bp fragment of the chicken LysRep element (GenBank # NW 060235) was amplified using PCR conditions known to one skilled in the art. Amplified PCR product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA).

Purified LysRep DNA was sequentially digested with restriction enzymes Not I and Xho I (5'end) and Mlu I and Apa I (3'end) (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Digested DNA was purified from restriction enzymes using a Zymo DNA Clean and Concentrator kit (Zymo Research). To insert the LysRep elements between the IS left and the MCS in pTnX-MCS (SEQ ID NO:2), the purified LysRep DNA and pTnX-MCS were digested with Not I and Xho I, purified as described above, and ligated using a Stratagene T4 Ligase Kit (Stratagene, Inc. La Jolla, CA) according to the manufacturer's protocol. Ligated product was transformed into *E. coli* Top10 competent cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to Invitrogen's protocol. Transformed bacteria were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) medium for 1 hour at

37°C before being spread to LB media (broth or agar) plates supplemented with 100 μg/ml ampicillin (LB/amp plates). These plates were incubated overnight at 37°C, and resulting colonies picked to LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in at least 250 ml of LB/amp broth and plasmid DNA harvested using a Qiagen Maxi-Prep Kit (column purification) according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). Column purified DNA was used as template for sequencing to verify the changes made in the vector were the desired changes and no further changes or mutations occurred. All sequencing was done on a Beckman Coulter CEQ 8000 Genetic Analysis System. Once a clone was identified that contained the 5' LysRep DNA, the vector was digested with Mlu I and Apa I as was the purified LysRep DNA. The same procedures described above were used to ligate the LysRep DNA into the backbone and verify that it was correct. Once a clone was identified that contained both LysRep elements, the DNA was isolated for use in cloning in specific genes of interest.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* containing the plasmid were grown in 500 mL aliquots of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight with shaking. Plasmid DNA was recovered from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 μL of PCR-grade water and stored at -20°C until needed.

g. *Preparation of Transposon-Based Vector TnPuro #5019 (p5019)*

This vector (SEQ ID NO:7) is a modification of p5012 (SEQ ID NO:4) described above in section 1.d. The modification includes insertion of the puromycin gene in the multiple cloning site adjacent to one of the HS4 insulator elements. To accomplish this ligation, the 602 bp puromycin gene was isolated from the vector pMOD Puro (Invivogen, Inc.) using PCR conditions known to one skilled in the art. Amplified PCR product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on a U.V. transilluminator. A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA).

Purified Puro DNA was digested with restriction enzyme Kas I (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Digested DNA was purified from restriction enzymes using a Zymo DNA Clean and Concentrator kit (Zymo Research). To insert the Puro gene into the MCS of p5012, the purified Puro DNA and p5012 were digested with Kas I, purified as described above, and ligated using a Stratagene T4 Ligase Kit (Stratagene, Inc. La Jolla, CA) according to the manufacturer's protocol. Ligated product was transformed into *E.*

coli Top10 competent cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to Invitrogen's protocol. Transformed bacteria were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) medium for 1 hour at 37°C before being spread to LB (broth or agar) plates supplemented with 100 μg/ml ampicillin (LB/amp plates). These plates were incubated overnight at 37°C and resulting colonies picked to LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in at least 250 ml of LB/amp broth and plasmid DNA harvested using a Qiagen Maxi-Prep Kit (column purification) according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). Column purified DNA was used as template for sequencing to verify the changes made in the vector were the desired changes and no further changes or mutations occurred. All sequencing was done on a Beckman Coulter CEQ 8000 Genetic Analysis System. Once a clone was identified that contained both Puro gene, the DNA was isolated for use in cloning in specific genes of interest.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* containing the plasmid were grown in 500 mL aliquots of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight with shaking. Plasmid DNA was recovered from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 μL of PCR-grade water and stored at -20°C until needed.

h. *Preparation of Transposon-Based Vector pTn-10 PuroMAR #5021 (p5021)*

This vector (SEQ ID NO:8) is a modification of p5018 (SEQ ID NO:5) described above in section 1.e. The modification includes insertion of the puromycin (puro) gene into the multiple cloning site adjacent to one of the MAR insulator elements. To accomplish this, the 602 bp puromycin gene was amplified by PCR from the vector pMOD Puro (Invitrogen Life Technologies, Carlsbad, CA). Amplified PCR product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA).

Purified DNA from the puromycin gene was digested with the restriction enzymes BsiWI and MluI (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Digested DNA was purified from agarose using a Zymo DNA Clean and Concentrator kit (Zymo Research). To insert the puro gene into the MCS of p5018, puro and p5018 were digested with BsiWI and MluI, purified as described above, and ligated using Stratagene's T4 Ligase Kit (La Jolla, CA) according to the manufacturer's protocol. Ligated product was transformed into *E.*

coli Top10 cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to the manufacturer's protocol. Transformed cells were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) for 1 hour at 37°C then spread onto LB agar plates supplemented with 100 μg/ml ampicillin (LB/amp plates). These plates were incubated overnight at 37°C. Resulting colonies were picked into LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in a minimum of 250 ml of LB/amp broth. The plasmid DNA was harvested using a Qiagen Maxi-Prep Kit according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). The DNA was used as a sequencing template to verify that the changes made in the vector were desired changes and that no further changes or mutations occurred. All sequencing was performed using Beckman Coulter's CEQ 8000 Genetic Analysis System. Once a clone was identified that contained the puro gene, the DNA was isolated and used for cloning in specific genes of interest.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* containing the plasmid of interest was grown in 500 ml of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight in a shaking incubator. Plasmid DNA was isolated from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 μL of PCR-grade water and stored at -20°C until needed.

i. *Preparation of Transposon-Based Vector TnGenMAR #5022 (p5022)*

This vector (SEQ ID NO:9) is a modification of p5021 (SEQ ID NO:8) described above under section 1.h. The modification includes insertion of the gentamycin gene in the multiple cloning site adjacent to one of the MAR insulator elements. To accomplish this ligation, the 1251 bp gentamycin gene was isolated from the vector pS65T-C1(ClonTech Laboratories, using PCR conditions known to one skilled in the art. Amplified PCR product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA).

Purified gentamycin DNA was digested with restriction enzyme BsiW I and Mlu I (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Digested DNA was purified from restriction enzymes using a Zymo DNA Clean and Concentrator kit (Zymo Research). To insert the gentamycin gene into the MCS of p5018, the purified gentamycin DNA and p5018 were digested with BsiW I and Mlu I, purified as described above, and ligated using a Stratagene T4 Ligase Kit (Stratagene, Inc. La Jolla, CA) according to the manufacturer's

protocol. Ligated product was transformed into *E. coli* Top10 competent cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to Invitrogen's protocol. Transformed bacteria were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) medium for 1 hour at 37°C before being spread to LB (broth or agar) plates supplemented with 100 μg/ml ampicillin (LB/amp plates). These plates were incubated overnight at 37°C, and resulting colonies picked to LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in at least 250 ml of LB/amp broth and plasmid DNA harvested using a Qiagen Maxi-Prep Kit (column purification) according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). Column purified DNA was used as template for sequencing to verify that the changes made in the vector were the desired changes and that no further changes or mutations occurred. All sequencing was done on a Beckman Coulter CEQ 8000 Genetic Analysis System. Once a clone was identified that contained both Puro gene, the DNA was isolated for use in cloning in specific genes of interest.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* containing the plasmid were grown in 500 mL aliquots of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight with shaking. Plasmid DNA was recovered from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 μL of PCR-grade water and stored at -20°C until needed.

j. *Preparation of Low Expression CMV Tn PuroMAR Flanked Backbone #5024 (p5024)*

This vector (SEQ ID NO:10) is a modification of p5018 (SEQ ID NO:5), which includes the deletion of the CMV Enhancer region of the transposase cassette. The CMV enhancer was removed from p5018 by digesting the backbone with MscI and AfeI restriction enzymes (New England Biolabs, Beverly, MA). The digested product was electrophoresed, stained with Syber Safe DNA Gel Stain (Invitrogen Life Technologies, Carlsbad, CA), and visualized on a Visi-Blue transilluminator (UVP Laboratory Products, Upland, CA). A band corresponding to the expected size of the backbone without the enhancer region was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA).

Backbone DNA from above was re-circularized using an Epicentre Fast Ligase Kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's protocol. The ligation was transformed into *E. coli* Top10 cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to the manufacturer's protocol. Transformed cells were incubated in 250 ml of SOC (GIBCO BRL, CAT# 15544-042) for 1 hour at 37°C then spread

onto LB agar plates supplemented with 100 μg/ml ampicillin (LB/amp plates). All plates were incubated overnight at 37°C. Resulting colonies were picked into LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in 5ml of LB/amp broth. Plasmid DNA was harvested using Fermentas' Gene Jet Plasmid Miniprep Kit according to the manufacturer's protocol (Glen Burnie, MD). The DNA was then used as a sequencing template to verify that any changes made in the vector were desired changes and that no further changes or mutations occurred. All sequencing was performed using Beckman Coulter's CEQ 8000 Genetic Analysis System. Once a clone was identified containing the replacement promoter fragment, the DNA was isolated and used for cloning in specific genes of interest.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* bacteria containing the plasmid of interest were grown in a minimum of 500 ml of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight in a shaking incubator. Plasmid DNA was isolated from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 μL of PCR-grade water and stored at -20°C until needed.

k. *Preparation of Low Expression CMV Tn PuroMAR Flanked Backbone #5025 (p5025)*

This vector (SEQ ID NO:11) is a modification of p5021 (SEQ ID NO:8), which includes the deletion of the CMV Enhancer of on the transposase cassette. The CMV enhancer was removed from p5021 by digesting the backbone with MscI and AfeI restriction enzymes (New England Biolabs, Beverly, MA). The digested product was electrophoresed, stained with Syber Safe DNA Gel Stain (Invitrogen Life Technologies, Carlsbad, CA), and visualized on a Visi-Blue transilluminator (UVP Laboratory Products, Upland, CA). A band corresponding to the expected size of the backbone without the enhancer region was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA).

Backbone DNA from above was re-circularized using an Epicentre Fast Ligase Kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's protocol. The ligation was transformed into *E. coli* Top10 cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to the manufacturer's protocol. Transformed cells were incubated in 250 ml of SOC (GIBCO BRL, CAT# 15544-042) for 1 hour at 37°C then spread onto LB (Luria-Bertani) agar plates supplemented with 100 μg/ml ampicillin (LB/amp plates). All plates were incubated overnight at 37°C. Resulting colonies were picked into LB/amp broth

for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in 5 ml of LB/amp broth. Plasmid DNA was harvested using Fermentas' Gene Jet Plasmid Miniprep Kit according to the manufacturer's protocol (Glen Burnie, MD). The DNA was then used as a sequencing template to verify that any changes made in the vector were desired changes and that no further changes or mutations occurred. All sequencing was performed using Beckman Coulter's CEQ 8000 Genetic Analysis System. Once a clone was identified containing the replacement promoter fragment, the DNA was isolated and used for cloning in specific genes of interest.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* bacteria containing the plasmid of interest were grown in a minimum of 500 ml of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight in a shaking incubator. Plasmid DNA was isolated from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 µL of PCR-grade water and stored at -20°C until needed.

1. *Preparation of Low Expression SV40 promoter Tn PuroMAR Flanked Backbone #5026 (p5026)*

This vector (SEQ ID NO:12) is a modification of p5018 (SEQ ID NO:5), which includes the replacement of the CMV Enhanced promoter of the transposase cassette, with the SV40 promoter from pS65T-C1 (Clontech, Mountainview, CA). The CMV enhanced promoter was removed from p5018 by digesting the backbone with MscI and AfeI restriction enzymes (New England Biolabs, Beverly, MA). The digested product was electrophoresed, stained with Syber Safe DNA Gel Stain (Invitrogen Life Technologies, Carlsbad, CA), and visualized on a Visi-Blue transilluminator (UVP Laboratory Products, Upland, CA). A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA). The SV40 promoter fragment was amplified to add the 5' and 3' cut sites, MscI and AscI, respectively. The PCR product was then cloned into pTopo Blunt II backbone (Invitrogen Life Technologies, Carlsbad, CA). Sequence verified DNA was then digested out of the pTopo Blunt II backbone (Invitrogen Life Technologies, Carlsbad, CA), with MscI and AfeI restriction enzymes (New England Biolabs, Beverly, MA). The digested product was electrophoresed, stained with Syber Safe DNA Gel Stain (Invitrogen Life Technologies, Carlsbad, CA), and visualized on a Visi-Blue transilluminator (UVP Laboratory Products, Upland, CA). A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA).

Purified digestion product was ligated into the excised backbone DNA using Epicentre's Fast Ligase Kit (Madison, WI) according to the manufacturer's protocol. The ligation product was transformed into *E. coli* Top10 cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to the manufacturer's protocol. Transformed cells were incubated in 250 ml of SOC (GIBCO BRL, CAT# 15544-042) for 1 hour at 37° C before then spread onto LB agar plates supplemented with 100 μg/ml ampicillin (LB/amp plates). All plates were incubated overnight at 37°C. Resulting colonies were picked into LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in 5 ml of LB/amp broth. The plasmid DNA was harvested using a Fermentas' Gene Jet Plasmid Miniprep Kit according to the manufacturer's protocol (Glen Burnie, MD). The DNA was then used as sequencing template to verify that any changes made in the vector were desired changes and that no further changes or mutations occurred. All sequencing was performed using Beckman Coulter's CEQ 8000 Genetic Analysis System. Once a clone was identified that contained the replacement promoter fragment, the DNA was isolated for use in cloning in specific genes of interest.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* bacteria containing the plasmid of interest were grown in a minimum of 500 mL of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight in a shaking incubator. Plasmid DNA was isolated from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 μL of PCR-grade water and stored at -20°C until needed.

m. *Preparation of Low Expression SV40 promoter Tn PuroMAR Flanked Backbone #5027 (p5027)*

This vector (SEQ ID NO:13) is a modification of p5021 (SEQ ID NO:8), which includes the replacement of the CMV Enhanced promoter of the transposase cassette, with the SV40 promoter from pS65T-C1 (Clontech, Mountainview, CA). The CMV enhanced promoter was removed from p5021 by digesting the backbone with MscI and AfeI restriction enzymes (New England Biolabs, Beverly, MA). The digested product was electrophoresed, stained with Syber Safe DNA Gel Stain (Invitrogen Life Technologies, Carlsbad, CA), and visualized on a Visi-Blue transilluminator (UVP Laboratory Products, Upland, CA). A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA). The SV40 promoter fragment was amplified to add the 5' and 3' cut sites, MscI and AscI, respectively. The PCR product was then cloned into pTopo

Blunt II backbone (Invitrogen Life Technologies, Carlsbad, CA). Sequence verified DNA was then digested out of the pTopo Blunt II backbone (Invitrogen Life Technologies, Carlsbad, CA), with MscI and AfeI restriction enzymes (New England Biolabs, Beverly, MA). The digested product was electrophoresed, stained with Syber Safe DNA Gel Stain (Invitrogen Life Technologies, Carlsbad, CA), and visualized on a Visi-Blue transilluminator (UVP Laboratory Products, Upland, CA). A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA).

Purified digestion product was ligated into the excised backbone DNA using Epicentre's Fast Ligase Kit (Madison, WI) according to the manufacturer's protocol. The ligation product was transformed into *E. coli* Top10 cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to the manufacturer's protocol. Transformed cells were incubated in 250 µl of SOC (GIBCO BRL, CAT# 15544-042) for 1 hour at 37°C before being spread onto LB agar plates supplemented with 100 µg/ml ampicillin (LB/amp plates). All plates were incubated overnight at 37°C. Resulting colonies were picked into LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on an ultraviolet transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in 5 ml of LB/amp broth. The plasmid DNA was harvested using a Fermentas' Gene Jet Plasmid Miniprep Kit according to the manufacturer's protocol (Glen Burnie, MD). The DNA was then used as sequencing template to verify that any changes made in the vector were desired changes and that no further changes or mutations occurred. All sequencing was performed using Beckman Coulter's CEQ 8000 Genetic Analysis System. Once a clone was identified that contained the replacement promoter fragment, the DNA was isolated for use in cloning in specific genes of interest.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* bacteria containing the plasmid of interest were grown in a minimum of 500 mL of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight in a shaking incubator. Plasmid DNA was isolated from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 µL of PCR-grade water and stored at -20°C until needed.

2. *Promoters*

A second embodiment of this invention are hybrid promoters that consist of elements from the constitutive CMV promoter and the estrogen inducible ovalbumin promoter. The goal of designing these promoters was to couple the high rate of expression associated with the CMV

promoter with the estrogen inducible function of the ovalbumin promoter. To accomplish this goal, two hybrid promoters, designated versions 1 and 2 (SEQ ID NOs:14 and 15, respectively)(Figure 1), were designed, built, and tested in cell culture using a gene other than a GH gene. Both versions 1 and 2 provided high rates of expression.

a. Version 1 CMV/Oval promoter 1 = ChOvp/CMVenh/CMVp

Hybrid promoter version 1 (SEQ ID NO:14) was constructed by ligating the chicken ovalbumin promoter regulatory elements to the 5' end of the CMV enhancer and promoter. A schematic is shown in Figure 1A.

Hybrid promoter version 1 was made by PCR amplifying nucleotides 1090 to 1929 of the ovalbumin promoter (GenBank # J00895) from the chicken genome and cloning this DNA fragment into the pTopo vector (Invitrogen, Carlsbad, CA). Likewise, nucleotides 245-918 of the CMV promoter and enhancer were removed from the pgWiz vector (ClonTech, Mountain View, CA) and cloned into the pTopo vector. By cloning each fragment into the multiple cloning site of the pTopo vector, an array of restriction enzyme sites were available on each end of the DNA fragments which greatly facilitated cloning without PCR amplification. Each fragment was sequenced to verify it was the correct DNA sequence. Once sequence verified, the pTopo clone containing the ovalbumin promoter fragment was digested with Xho I and EcoR I, and the product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA). The pTopo clone containing the CMV promoter was treated in the same manner to open up the plasmid 5' to the CMV promoter; these restriction enzymes also allowed directional cloning of the ovalbumin promoter fragment upstream of CMV.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* containing the plasmid were grown in 500 mL aliquots of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight with shaking. Plasmid DNA was recovered from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 µL of PCR-grade water and stored at -20°C until needed.

b. Version 2 CMV/Oval promoter = ChSDRE/CMVenh/ChNRE/CMVp

Hybrid promoter version 2 (SEQ ID NO:15) consisted of the steroid dependent response element (SDRE) ligated 5' to the CMV enhancer (enh) and the CMV enhancer and promoter separated by the chicken ovalbumin negative response element (NRE).

A schematic is shown in Figure 1B. Hybrid promoter version 2 was made by PCR amplifying the SDRE, nucleotides 1100 to 1389, and nucleotides 1640 to 1909 of the negative response element (NRE) of the ovalbumin promoter (GenBank # J00895) from the chicken

genome and cloning each DNA fragment into the pTopo vector. Likewise, nucleotides 245-843 of the CMV enhancer and nucleotides 844-915 of the CMV promoter were removed from the pgWiz vector and each cloned into the pTopo vector. By cloning each piece into the multiple cloning site of the pTopo vector, an array of restriction enzyme sites were available on each end of the DNA fragments which greatly facilitated cloning without PCR amplification.

Each fragment was sequenced to verify it was the correct DNA sequence. Once sequence verified, the pTopo clone containing the ovalbumin SDRE fragment was digested with Xho I and EcoR I to remove the SDRE, and the product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA). The pTopo clone containing the CMV enhancer was treated in the same manner to open up the plasmid 5' to the CMV enhancer; these restriction enzymes also allowed directional cloning of the ovalbumin SDRE fragment upstream of CMV. The ovalbumin NRE was removed from pTopo using NgoM IV and Kpn I; the same restriction enzymes were used to digest the pTopo clone containing the CMV promoter to allow directional cloning of the NRE.

The DNA fragments were purified as described above. The new pTopo vectors containing the ovalbumin SDRE/CMV enhancer and the NRE/CMV promoter were sequence verified for the correct DNA sequence. Once sequence verified, the pTopo clone containing the ovalbumin SDRE/CMV enhancer fragment was digested with Xho I and NgoM IV to remove the SDRE/CMV Enhancer, and the product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA). The pTopo clone containing the NRE/CMVpromoter was treated in the same manner to open up the plasmid 5' to the CMV enhancer. These restriction enzymes also allowed directional cloning of the ovalbumin SDRE fragment upstream of CMV. The resulting promoter hybrid was sequence verified to insure that it was correct.

All plasmid DNA was isolated by standard procedures. Briefly, *E. coli* containing the plasmid were grown in 500 mL aliquots of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight with shaking. Plasmid DNA was recovered from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 µL of PCR-grade water and stored at -20°C until needed.

c. Version 3 Promoter CMV/Oval promoter = CMVenh/ChOvp/CMVp

Hybrid promoter version 3 is shown at base pairs 4937 to 6465 of SEQ ID NO:19. This promoter consisted of the CMV enhancer and promoter being separated by the regulatory elements of the chicken ovalbumin promoter (OVp). Due to the size of the ovalbumin promoter elements separating the CMV enhancer and promoter, it was expected that protein expression would be decreased with this promoter. This was indeed the case in cell culture.

3. *Transposases and Insertion Sequences*

In a further embodiment of the present invention, the transposase found in the transposase-based vector is an altered target site (ATS) transposase and the insertion sequences are those recognized by the ATS transposase. However, the transposase located in the transposase-based vectors is not limited to a modified ATS transposase and can be derived from any transposase. Transposases known in the prior art include those found in AC7, Tn5SEQ1, Tn916, Tn951, Tn1721, Tn 2410, Tn1681, Tn1, Tn2, Tn3, Tn4, Tn5, Tn6, Tn9, Tn10, Tn30, Tn101, Tn903, Tn501, Tn1000 (γδ), Tn1681, Tn2901, AC transposons, Mp transposons, Spm transposons, En transposons, Dotted transposons, Mu transposons, Ds transposons, dSpm transposons and I transposons. According to the present invention, these transposases and their regulatory sequences are modified for improved functioning as follows: a) the addition one or more modified Kozak sequences comprising any one of SEQ ID NOs:28 to 37 at the 3' end of the promoter operably-linked to the transposase; b) a change of the codons for the first several amino acids of the transposase, wherein the third base of each codon was changed to an A or a T without changing the corresponding amino acid; c) the addition of one or more stop codons to enhance the termination of transposase synthesis; and/or, d) the addition of an effective polyA sequence operably-linked to the transposase to further enhance expression of the transposase gene.

Although not wanting to be bound by the following statement, it is believed that the modifications of the first several N-terminal codons of the transposase gene increase transcription of the transposase gene, in part, by increasing strand dissociation. It is preferable that between approximately 1 and 20, more preferably 3 and 15, and most preferably between 4 and 12 of the first N-terminal codons of the transposase are modified such that the third base of each codon is changed to an A or a T without changing the encoded amino acid. In one embodiment, the first ten N-terminal codons of the transposase gene are modified in this manner. It is also preferred that the transposase contain mutations that make it less specific for preferred insertion sites and thus increases the rate of transgene insertion as discussed in U.S. Patent No. 5,719,055.

In some embodiments, the transposon-based vectors are optimized for expression in a particular host by changing the methylation patterns of the vector DNA. For example, prokaryotic methylation may be reduced by using a methylation deficient organism for

production of the transposon-based vector. The transposon-based vectors may also be methylated to resemble eukaryotic DNA for expression in a eukaryotic host.

Transposases and insertion sequences from other analogous eukaryotic transposon-based vectors that can also be modified and used are, for example, the Drosophila P element derived vectors disclosed in U.S. Patent No. 6,291,243; the Drosophila mariner element described in Sherman et al. (1998); or the sleeping beauty transposon. See also Hackett et al. (1999); D. Lampe et al., 1999. Proc. Natl. Acad. Sci. USA, 96:11428-11433; S. Fischer et al., 2001. Proc. Natl. Acad. Sci. USA, 98:6759-6764; L. Zagoraiou et al., 2001. Proc. Natl. Acad. Sci. USA, 98:11474-11478; and D. Berg et al. (Eds.), Mobile DNA, Amer. Soc. Microbiol. (Washington, D.C., 1989). However, it should be noted that bacterial transposon-based elements are preferred, as there is less likelihood that a eukaryotic transposase in the recipient species will recognize prokaryotic insertion sequences bracketing the transgene.

Many transposases recognize different insertion sequences, and therefore, it is to be understood that a transposase-based vector will contain insertion sequences recognized by the particular transposase also found in the transposase-based vector. In a preferred embodiment of the invention, the insertion sequences have been shortened to about 70 base pairs in length as compared to those found in wild-type transposons that typically contain insertion sequences of well over 100 base pairs.

While the examples provided below incorporate a "cut and insert" Tn10 based vector that is destroyed following the insertion event, the present invention also encompasses the use of a "rolling replication" type transposon-based vector. Use of a rolling replication type transposon allows multiple copies of the transposon/transgene to be made from a single transgene construct and the copies inserted. This type of transposon-based system thereby provides for insertion of multiple copies of a transgene into a single genome. A rolling replication type transposon-based vector may be preferred when the promoter operably-linked to gene of interest is endogenous to the host cell and present in a high copy number or highly expressed. However, use of a rolling replication system may require tight control to limit the insertion events to non-lethal levels. Tn1, Tn2, Tn3, Tn4, Tn5, Tn9, Tn21, Tn501, Tn551, Tn951, Tn1721, Tn2410 and Tn2603 are examples of a rolling replication type transposon, although Tn5 could be both a rolling replication and a cut and insert type transposon.

4. *Other Promoters and Enhancers*

The first promoter operably-linked to the transposase gene and the second promoter operably-linked to the gene of interest can be a constitutive promoter or an inducible promoter. Constitutive promoters include, but are not limited to, immediate early cytomegalovirus (CMV) promoter, herpes simplex virus 1 (HSV1) immediate early promoter, SV40 promoter, lysozyme

promoter, early and late CMV promoters, early and late HSV promoters, β-actin promoter, tubulin promoter, Rous-Sarcoma virus (RSV) promoter, and heat-shock protein (HSP) promoter. Inducible promoters include tissue-specific promoters, developmentally-regulated promoters and chemically inducible promoters. Examples of tissue-specific promoters include the glucose-6-phosphatase (G6P) promoter, vitellogenin promoter, ovalbumin promoter, ovomucoid promoter, conalbumin promoter, ovotransferrin promoter, prolactin promoter, kidney uromodulin promoter, and placental lactogen promoter. The G6P promoter sequence may be deduced from a rat G6P gene untranslated upstream region provided in GenBank accession number U57552.1. Examples of developmentally-regulated promoters include the homeobox promoters and several hormone induced promoters. Examples of chemically inducible promoters include reproductive hormone induced promoters and antibiotic inducible promoters such as the tetracycline inducible promoter and the zinc-inducible metallothionine promoter.

Other inducible promoter systems include the Lac operator repressor system inducible by IPTG (isopropyl beta-D-thiogalactoside) (Cronin, A. et al. 2001. Genes and Development, v. 15), ecdysone-based inducible systems (Hoppe, U. C. et al. 2000. Mol. Ther. 1:159-164); estrogen-based inducible systems (Braselmann, S. et al. 1993. Proc. Natl. Acad. Sci. 90:1657-1661); progesterone-based inducible systems using a chimeric regulator, GLVP, which is a hybrid protein consisting of the GAL4 binding domain and the herpes simplex virus transcriptional activation domain, VP16, and a truncated form of the human progesterone receptor that retains the ability to bind ligand and can be turned on by RU486 (Wang, et al. 1994. Proc. Natl. Acad. Sci. 91:8180-8184); CID-based inducible systems using chemical inducers of dimerization (CIDs) to regulate gene expression, such as a system wherein rapamycin induces dimerization of the cellular proteins FKBP12 and FRAP (Belshaw, P. J. et al. 1996. J. Chem. Biol. 3:731-738; Fan, L. et al. 1999. Hum. Gene Ther. 10:2273-2285; Shariat, S.F. et al. 2001. Cancer Res. 61:2562-2571; Spencer, D.M. 1996. Curr. Biol. 6:839-847). Chemical substances that activate the chemically inducible promoters can be administered to the animal containing the transgene of interest via any method known to those of skill in the art.

Other examples of cell-specific and constitutive promoters include but are not limited to smooth-muscle SM22 promoter, including chimeric SM22alpha/telokin promoters (Hoggatt A.M. et al., 2002. Circ Res. 91(12):1151-9); ubiquitin C promoter (Biochim Biophys Acta, 2003. Jan. 3;1625(1):52-63); Hsf2 promoter; murine COMP (cartilage oligomeric matrix protein) promoter; early B cell-specific mb-1 promoter (Sigvardsson M., et al., 2002. Mol. Cell Biol. 22(24):8539-51); prostate specific antigen (PSA) promoter (Yoshimura I. et al., 2002, J. Urol. 168(6):2659-64); exorh promoter and pineal expression-promoting element (Asaoka Y., et al., 2002. Proc. Natl. Acad. Sci. 99(24):15456-61); neural and liver ceramidase gene promoters (Okino N. et al.,

2002. Biochem. Biophys. Res. Commun. 299(1):160-6); PSP94 gene promoter/enhancer (Gabril M.Y. et al., 2002. Gene Ther. 9(23):1589-99); promoter of the human FAT/CD36 gene (Kuriki C., et al., 2002. Biol. Pharm. Bull. 25(11):1476-8); VL30 promoter (Staplin W.R. et al., 2002. Blood October 24, 2002); and, IL-10 promoter (Brenner S., et al., 2002. J. Biol. Chem. December 18, 2002). Additional promoters are shown in Table 1.

Examples of avian promoters include, but are not limited to, promoters controlling expression of egg white proteins, such as ovalbumin, ovotransferrin (conalbumin), ovomucoid, lysozyme, ovomucin, g2 ovoglobulin, g3 ovoglobulin, ovoflavoprotein, ovostatin (ovomacroglobin), cystatin, avidin, thiamine-binding protein, glutamyl aminopeptidase minor glycoprotein 1, minor glycoprotein 2; and promoters controlling expression of egg-yolk proteins, such as vitellogenin, very low-density lipoproteins, low density lipoprotein, cobalamin-binding protein, riboflavin-binding protein, biotin-binding protein (Awade, 1996. Z. Lebensm. Unters. Forsch. 202:1-14). An advantage of using the vitellogenin promoter is that it is active during the egg-laying stage of an animal's life-cycle, which allows for the production of the protein of interest to be temporally connected to the import of the protein of interest into the egg yolk when the protein of interest is equipped with an appropriate targeting sequence. As used herein, the "protein of interest" is GH. In some embodiments, the avian promoter is an oviduct-specific promoter. As used herein, the term "oviduct-specific promoter" includes, but is not limited to, ovalbumin; ovotransferrin (conalbumin); ovomucoid; 01, 02, 03, 04 or 05 avidin; ovomucin; g2 ovoglobulin; g3 ovoglobulin; ovoflavoprotein; and ovostatin (ovomacroglobin) promoters.

When germline transformation occurs via cardiovascular, intraovarian or intratesticular administration, or when hepatocytes are targeted for incorporation of components of a vector through non-germ line administration, liver-specific promoters may be operably-linked to the gene of interest to achieve liver-specific expression of the transgene. Liver-specific promoters of the present invention include, but are not limited to, the following promoters, vitellogenin promoter, G6P promoter, cholesterol-7-alpha-hydroxylase (CYP7A) promoter, phenylalanine hydroxylase (PAH) promoter, protein C gene promoter, insulin-like growth factor I (IGF-I) promoter, bilirubin UDP-glucuronosyltransferase promoter, aldolase B promoter, furin promoter, metallothionine promoter, albumin promoter, and insulin promoter.

Also included in this invention are modified promoters/enhancers wherein elements of a single promoter are duplicated, modified, or otherwise changed. In one embodiment, steroid hormone-binding domains of the ovalbumin promoter are moved from about -3.5 kb to within approximately the first 1000 base pairs of the gene of interest. Modifying an existing promoter with promoter/enhancer elements not found naturally in the promoter, as well as building an

entirely synthetic promoter, or drawing promoter/enhancer elements from various genes together on a non-natural backbone, are all encompassed by the current invention.

Accordingly, it is to be understood that the promoters contained within the transposon-based vectors of the present invention may be entire promoter sequences or fragments of promoter sequences. The constitutive and inducible promoters contained within the transposon-based vectors may also be modified by the addition of one or more modified Kozak sequences comprising any one of SEQ ID NOs:40 to 49.

As indicated above, the present invention includes transposon-based vectors containing one or more enhancers. These enhancers may or may not be operably-linked to their native promoter and may be located at any distance from their operably-linked promoter. A promoter operably-linked to an enhancer and a promoter modified to eliminate repressive regulatory effects are referred to herein as an "enhanced promoter." The enhancers contained within the transposon-based vectors may be enhancers found in birds, such as an ovalbumin enhancer, but are not limited to these types of enhancers. In one embodiment, an approximately 675 base pair enhancer element of an ovalbumin promoter is cloned upstream of an ovalbumin promoter with 300 base pairs of spacer DNA separating the enhancer and promoter. In one embodiment, the enhancer used as a part of the present invention comprises base pairs 1-675 of a chicken ovalbumin enhancer from GenBank accession #S82527.1. The polynucleotide sequence of this enhancer is provided in SEQ ID NO:50.

Also included in some of the transposon-based vectors of the present invention are cap sites and fragments of cap sites. In one embodiment, approximately 50 base pairs of a 5' untranslated region wherein the capsite resides are added on the 3' end of an enhanced promoter or promoter. An exemplary 5' untranslated region is provided in SEQ ID NO:51. A putative cap-site residing in this 5' untranslated region preferably comprises the polynucleotide sequence provided in SEQ ID NO:52.

In one embodiment of the present invention, the first promoter operably-linked to the transposase gene is a constitutive promoter and the second promoter operably-linked to the gene of interest is a cell specific promoter. In the second embodiment, use of the first constitutive promoter allows for constitutive activation of the transposase gene and incorporation of the gene of interest into virtually all cell types, including the germline of the recipient animal. Although the gene of interest is incorporated into the germline generally, the gene of interest may only be expressed in a tissue-specific manner to achieve gene therapy. A transposon-based vector having a constitutive promoter operably-linked to the transposase gene can be administered by any route, and in several embodiments, the vector is administered to the cardiovascular system, directly to an ovary, to an artery leading to the ovary or to a lymphatic system or fluid proximal to the ovary.

In another embodiment, the transposon-based vector having a constitutive promoter operably-linked to the transposase gene can be administered to vessels supplying the liver, muscle, brain, lung, kidney, heart or any other desired organ, tissue or cellular target. In another embodiment, the transposon-based vector having a constitutive promoter operably-linked to the transposase gene can be administered to cells for culture *in vitro*.

It should be noted that cell- or tissue-specific expression as described herein does not require a complete absence of expression in cells or tissues other than the preferred cell or tissue. Instead, "cell-specific" or "tissue-specific" expression refers to a majority of the expression of a particular gene of interest in the preferred cell or tissue, respectively.

When incorporation of the gene of interest into the germline is not preferred, the first promoter operably-linked to the transposase gene can be a tissue-specific or cell-specific promoter. For example, transfection of a transposon-based vector containing a transposase gene operably-linked to a liver specific promoter such as the G6P promoter or vitellogenin promoter provides for activation of the transposase gene and incorporation of the gene of interest in the cells of the liver *in vivo*, or *in vitro*, but not into the germline and other cells generally. In another example, transfection of a transposon-based vector containing a transposase gene operably-linked to an oviduct specific promoter such as the ovalbumin promoter provides for activation of the transposase gene and incorporation of the gene of interest in the cells of the oviduct *in vivo* or into oviduct cells *in vitro*, but not into the germline and other cells generally. In this embodiment, the second promoter operably-linked to the gene of interest can be a constitutive promoter or an inducible promoter. In one embodiment, both the first promoter and the second promoter are an ovalbumin promoter. In embodiments wherein tissue-specific expression or incorporation is desired, it is preferred that the transposon-based vector is administered directly to the tissue of interest, to the cardiovascular system which provides blood supply to the tissue of interest, to an artery leading to the organ or tissue of interest or to fluids surrounding the organ or tissue of interest. In one embodiment, the tissue of interest is the oviduct and administration is achieved by direct injection into the oviduct, into the cardiovascular system, or an artery leading to the oviduct. In another embodiment, the tissue of interest is the liver and administration is achieved by direct injection into the cardiovascular system, the portal vein or hepatic artery. In another embodiment, the tissue of interest is cardiac muscle tissue in the heart and administration is achieved by direct injection into the coronary arteries or left cardiac ventricle. In another embodiment, the tissue of interest is neural tissue and administration is achieved by direct injection into the cardiovascular system, the left cardiac ventricle, a cerebrovascular or spinovascular artery. In yet another embodiment, the target is a solid tumor and the

administration is achieved by injection into a vessel supplying the tumor or by injection into the tumor.

Accordingly, cell specific promoters may be used to enhance transcription in selected tissues. In birds, for example, promoters that are found in cells of the fallopian tube, such as ovalbumin, conalbumin, ovomucoid and/or lysozyme, are used in the vectors to ensure transcription of the gene of interest in the epithelial cells and tubular gland cells of the fallopian tube, leading to synthesis of the desired protein encoded by the gene and deposition into the egg white. In liver cells, the G6P promoter may be employed to drive transcription of the gene of interest for protein production. Proteins made in the liver of birds may be delivered to the egg yolk. Proteins made in transfected cells in vitro may be released into cell culture medium.

In order to achieve higher or more efficient expression of the transposase gene, the promoter and other regulatory sequences operably-linked to the transposase gene may be those derived from the host. These host specific regulatory sequences can be tissue specific as described above or can be of a constitutive nature.

Table 1

Reproductive tissue	**Promoter**	**Ref.**	**Function/comments**
testes, spermatogenesis	SPATA4	1	constitutive 30 d after birth in rat
placenta, glycoprotein	ERVWE1	2	URE, Upstream Regulatory Element is tissue spec. enhancer
breast epithelium and breast cancer	mammaglobin	6	specific to breast epithelium and cancer
prostate	EPSA	17	enhanced prostate-specific antigen promoter
testes	ATC	25	AlphaT-catenin specific for testes, skeletal, brain cardiomyocytes
prostate	PB	67	probasin promoter
Vision			
rod/cone	mCAR	3	cone photoreceptors and pinealocytes
retina	ATH5	15	functions in retinal ganglia and precursors
eye, brain	rhodopsin	27	
kertocytes	keratocan	42	specific to the corneal stroma
retina	RPE65	59	
Muscle			
vascular smooth muscle	TFPI	13	Tissue Factor Pathway Inhibitor – low level expression in endothelial and smooth muscle cells of vascular system
cardiac specific	MLC2v	14, 26	ventricular myosin light chain
cardiac	CAR3	18	BMP response element that directs cardiac specific expression
skeletal	C5-12	22	high level, muscle spec expression to drive target gene
skeletal	AdmDys,	32	muscle creatine kinase promoter

Reproductive tissue	Promoter	Ref.	Function/comments
	AdmCTLA4Ig		
smooth muscle	PDE5A	41	chromosome 4q26, phosphodiesterase use intronic splicing elements to restrict expression to smooth
smooth muscle	AlphaTM	45	muscle vs skeletal
skeletal	myostatin	48	fiber type-specific expression of myostatin
Endocrine/nervous			
glucocorticoid	GR 1B-1E	4, 12	glucocorticoid receptor promoter/ all cells
neuroblastoma	M2-2	8, 36	M2 muscarinic receptor amyloid beta-protein; 30 bp fragment
brain	Abeta	16	needed for PC12 and glial cell expression neuron-specific; high in hippocampus,
brain	enolase	21	intermediate in cortex, low in cerebellum clusters acetylcholine receptors at
synapses	rapsyn	29	neuromuscular junction express limited to neurons in central and peripheral nervous system and specific endocrine cells in adenohypophysis,
neuropeptide precursor	VGF	39	adrenal medulla, GI tract and pancreas use of methylation to control tissue
mammalian nervous system	BMP/RA	46	specificity in neural cells.
central and peripheral noradrenergic neurons	Phox2a/Phox2b	47	regulation of neuron differentiation
brain	BAI1-AP4	55	spec to cerebral cortex and hippocampus
Gastrointestinal			
UDP glucoronsyltransferase	UGT1A7	11	gastric mucosa
	UGT1A8	11	small intestine and colon
	UGT1A10	11	small intestine and colon
colon cancer	PKCbetaII	20	Protein kinase C betaII (PKCbetaII); express in colon cancer to selectively kill it.
Cancer			
tumor suppressor 4.1B	4.1B	5	2 isoforms, 1 spec to brain, 1 in kidney
nestin	nestin	63	second intron regulates tissue specificity
cancer spec promoter	hTRT/hSPA1	68	dual promoter system for cancer specificity
Blood/lymph system			
Thyroid	thyroglobulin	10	Thyroid spec. -- express to kill thyroid tumors
Thyroid	calcitonin	10	medullary thyroid tumors
Thyroid	GR 1A	12	regulation controlled by DREAM
thyroid	thyroglobulin	50	transcriptional repressor
arterial endothelial cells	ALK1	60	activin receptor-like kinase
Nonspecific			
RNA polymerase II		7	

Reproductive tissue	Promoter	Ref.	Function/comments
gene silencing	Gnasx1, Nespas	31	
beta-globin	beta globin	53	
Cardiac	M2-1	8	M2 muscarinic receptor
Lung	hBD-2	19	IL-17 induced transcription in airway epithelium
pulmonary surfactant protein	SP-C	62	Alveolar type II cells
ciliated cell-specific prom	FOZJ1	70	use in ciliated epithelial cells for CF treatment
surfactant protein expression	SPA-D	73	Possible treatment in premature babies
Clara cell secretory protein	CCSP	75	
Dental			
teeth/bone	DSPP	28	extracellular matrix protein dentin sialophosphoprotein
Adipose			
adipogenesis	EPAS1	33	endothelial PAS domain -- role in adipocyte differentiation
Epidermal			
differentiated epidermis	involucrin	38	
desmosomal protein	CDSN	58	stratum granulosum and stratum corneum of epidermis
Liver			
liver spec albumin	Albumin	49	
serum alpha-fetoprotein	AFP	56	liver spec regulation

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4. *Vectors for hGH Production*

The vectors of the present invention employ some of the vector components (backbone vectors and promoters) described in the previous section and also include the multiple cloning site (MCS) comprising the gene of interest. In one embodiment, the gene of interest encodes GH. In one embodiment the gene of interest encodes human (h)GH. The vectors SEQ ID NO:17 through 39 are all provided in the Appendix and all contain a gene of interest encoding hGH.

B. Methods of Transfecting Cells

1. *Transfection of LMH2A and LMH Cells in vitro*

DNA

GH expression vector DNA (*e.g.*, any one of SEQ ID NOs:17-39) DNA was prepared in either methylating or non-methylating bacteria, and was endotoxin-free. Agarose gels showed a single plasmid of the appropriate size. DNA was resuspended in molecular biology grade, sterile water at a concentration of at least 0.5 μg/μl. The concentration was verified by spectrophotometry, and the 260/280 ratio was 1.8 or greater. A stock of each DNA sample, diluted to 0.5ug/ul in sterile, molecular biology grade water, was prepared in the cell culture lab, and this stock used for all transfections. When not in use, the DNA stocks were kept frozen at -30^{0}C in small aliquots to avoid repeated freezing and thawing.

Transfection

The transfection reagent used for LMH2A or LMH cells was FuGENE 6 (Roche Applied Science). This reagent was used at a 1:6 ratio (ug of DNA: ul of transfection reagent) for all transfections in LMH2A or LMH cells. The chart below shows the amount of DNA and FuGENE 6 used for typical cell culture formats (T25 and T75 tissue culture flasks). If it is necessary to perform transfections in other formats, the amounts of serum free medium (SFM), FuGENE 6 and DNA are scaled appropriately based on the surface area of the flask or well used. The diluent (SFM) is any serum-free cell culture media appropriate for the cells and it did not contain any antibiotics or fungicides.

Table 2

DNA:FuGENE = 1:6 [DNA]=0.5μg/μl

	T25	T75
SFM	250 μl	800 μl
FuGENE 6	12 μl	48 μl
DNA	4 μl	16 μl

1. Cells used for transfection were split 24-48 hours prior to the experiment, so that they were actively growing and 50-80% confluent at the time of transfection.

2. FuGENE was warmed to room temperature before use. Because FuGENE is sensitive to prolonged exposure to air, the vial was kept tightly closed when not in use. The vial of FuGENE was returned to the refrigerator as soon as possible.

3. The required amount of FuGENE was pipetted into the SFM in a sterile microcentrifuge tube. The fluid was mixed gently but thoroughly, by tapping or flicking the tube, and incubated for 5 minutes at room temperature.

4. The required amount of DNA was added to the diluted FuGENE and mixed by vortexing for one second.

5. The mixture was incubated at room temperature for 1 hour.

6. During the incubation period, media on cells was replaced with fresh growth media. This media optionally contained serum, if needed, but did not contain antibiotics or fungicides unless absolutely required, as this can reduce the transfection efficiency.

7. The entire volume of the transfection complex was added to the cells. The flask was rocked to mix thoroughly.

8. The flasks were incubated at 37°C and 5% CO_2.

9. Cells were fed and samples obtained as required. After the first 24 hours, cells were optionally fed with media containing antibiotics and/or fungicides, if desired.

2. *Transfection of Other Cells*

The same methods described above for LMH and LMH2A cells are used for transfection of chicken tubular gland cells or other cell types such as Chinese hamster ovary (CHO) cells, CHO-K1 cells, chicken embryonic fibroblasts, HeLa cells, Vero cells, FAO (liver cells), human 3T3 cells, A20 cells, EL4 cells, HepG2 cells, J744A cells, Jurkat cells, P388D1 cells, RC-4B/c cells, SK-N-SH cells, Sp2/mIL-6 cells, SW480 cells, 3T6 Swiss cells, human myeloma cells, and human ARPT-19 cells.

C. Production and Purification of the Recombinant Fusion Protein, 3xFlag-hGH, Produced by the Transfected Cells

1. *Media preparation.*

Media containing recombinant 3xFlag-hGH produced by transfected cells was harvested and immediately frozen. Later the medium was thawed, filtered through a 0.45 micron cellulose acetate bottle-top filter to ensure that all particulate was removed prior to being loaded on the column.

2. *Affinity Purification*

The medium containing recombinant 3xFlag-hGH produced by transfected cells was subjected to affinity purification using an Anti-Flag M2 Affinity Gel (Sigma, product code A2220) loaded onto a Poly-Prep Chromatography Columns (BioRad, catalog 731-1550). A slurry of anti-flag M2 gel was applied to Poly-Prep Chromatography Column and the column was equilibrated at 1 ml/min with wash buffer (Tris Buffered Saline (TBS)) for 30 column volumes. After equilibration was complete, the prepared medium containing 3xFlag-hGH from cultured and transfected cells was applied to the column.

The media sample passed through the column, and the column was washed for 10 column volumes with TBS. Next, 8 column volumes elution buffer (100 mM Tris, 0.5M NaCl, pH 2.85) were run through the column, followed by 4 column volumes of TBS and the eluent was collected. The eluent was transferred to an Amicon Ultra-15 (that was pre-washed with TBS) and centrifuged at 3,500 x g until the sample was concentrated to the desired volume.

3. *Size exclusion chromatography*

The concentrated eluent from the affinity purification procedure was then subjected to size exclusion chromatography as a final step in the purification procedure. First, a superdex 200 10/300 GL column (GE Healthcare) was equilibrated with TBS. Multiple size exclusion runs were done in which a sample volume of 250µl for each run was passed over the column. Fractions containing 3xFlag-hGH from each run were then pooled, transferred to an Amicon Ultra-15, and concentrated to the desired final volume.

The purification procedure was evaluated at various stages using a sandwich ELISA assay. SDS-PAGE analysis with subsequent Coomassie blue staining was done to indicate both molecular weight and purity of the purified 3xFlag-hGH.

4. *Measurement of purified 3xFlag-hGH*

The purified 3xFlag-hGH was quantitated using: densitometry on a Coomassie stained SDS-PAGE gel using known concentrations of hGH standard; densitometry on Western Blots using known concentrations of hGH standard and/or 3xFlag-alkaline phosphatase; and indirect ELISA using known concentrations of hGH standard.

5. Enterokinase digestion of 3xFlag-hGH

In order to produce the mature hGH from the purified recombinant 3xFlag-hGH, the amino-terminal 3xFlag epitope was removed by enterokinase digestion. Recombinant enterokinase (Novagen) was added to the purified 3xFlag-hGH at a ratio of 1.0 Unit of enterokinase to 50 μg of 3xFlag-hGH. The reaction was incubated at room temperature for 16 hours with gentle agitation. Removal of the 3xFlag epitope was evidenced by a band shift on a Coomassie stained SDS-PAGE gel in which the enterokinase digested 3xFlag-hGH migrated at a lower molecular weight relative to the undigested 3xFlag-hGH. Additionally, Western blot analysis indicated that the 3xFlag epitope was no longer present on the enterokinase digested 3xFlag-hGH when the blot was probed against anti-flag immunoglobulins.

D. 3xFlag-hGH Detection

1. 3xFlag-hGH Measurement with ELISA

3xFlag-hGH was measured using the following sandwich ELISA protocol:

1. Diluted monoclonal anti-hGH (Biodesign International, Cat. # E45902M) 1:1000 in 2x-carbonate, pH 9.6 such that the final working dilution concentration is 3.13 μg/mL.
2. Added 100 uL of the diluted antibody into to the appropriate wells of the ELISA plate.
3. Allowed 96-well plate to coat overnight at 4°C or for 1 hr at 37°C.
4. Washed the ELISA plate five times with wash buffer (1x TBS/0.05% TWEEN).
5. Transferred 200 μL of blocking buffer (1.5% BSA/1x TBS/0.05% TWEEN) to the appropriate wells of the ELISA plate and allowed 96-well plate to block overnight @ 4°C or for 45 minutes at room temperature.
6. Diluted the purified fusion 3xFlag-hGH standard in negative control media (5% FCS/Waymouth, Gibco) such that the final working dilution concentration was 16 ng/mL.
7. Dilute test samples in negative control media (5% FCS/Waymouth , Gibco).
8. Removed the blocking buffer by manually "flicking" the ELISA plate into the sink.
9. Added the diluted samples and fusion protein standards into 96-well plate and incubated the ELISA plate at room temperature for 1 hour.
10. Diluted fresh Anti FLAG M2 Alkaline Phosphatase Antibody 1:8,000 (Sigma, Cat. # A9469) such that the final working dilution concentration was 125 ng/mL.
11. Added 100 μL of the diluted antibody into to the appropriate wells of the ELISA plate.
12. Incubated the ELISA plate at room temperature for 1 hour.
13. Diluted the p-nitrophenyl phosphate substrate solution in 1X DEA substrate buffer, pH 9.8 (KPL, Cat.# 50-80-02) such that the final working dilution concentration is 1 mg/mL.
14. Washed the ELISA plate five times with wash buffer (1x TBS/0.05% TWEEN).

15. Added 100 μL of the diluted p-nitrophenyl phosphate substrate solution to the appropriate wells of the ELISA plate

16. Using plate reader, took the absorbance readings at 405 nm of the ELISA plate at 30, 60, 90, and 120 minute intervals.

Culture medium was applied to the ELISA either in an undiluted or slightly diluted manner. 3xFlag-hGH is detected in this assay. The 3xFlag-hGH levels were determined by reference to the 3xFlag-hGH standard curve and are presented in various figures throughout this application.

2. *3xFlag-hGH Detection with Immunocytochemistry*

Transfected cells were fixed and examined using immunohostochemical techniques known to one of ordinary skill in the art. 3xFlag-hGH immunoreactivity was observed in the perinuclear cytoplasm of transfected LMH2a cells. Preabsorption of the anti-GH antiserum with GH prevented staining of the transfected cells.

Immunoreactivity to a 3xFlag-hGH fusion protein was detected in transfected tubular gland cells using anti-3x flag-FITC primary antiserum.

3. *3xFlag-hGH Production*

Several experiments were conducted to compare the backbone vectors containing the same gene of interest. For GH expression in tubular gland cells, significant differences were observed when protein expression was driven by the ovalbumin promoter. For example, the ovalbumin-hGH gene in TnMAR (SEQ ID NO:26) expressed at a rate 25% to 40% more than the same gene construct in the TnHS4 vector (SEQ ID NO:24) but was only 5% better than the same gene construct in the LysRep vs 2 vector (SEQ ID NO:25).

For protein expression in cultures of LMH2A cells, we developed a vector that allows selection of the cells expressing only the protein of interest. This selection vector contains a gene expressing the antibiotic puromycin, an antibiotic that is toxic to eukaryotic cells. The gene is contained within the insertion sequences of the transposon gene, so it is inserted with the Transposon gene and gene of interest. The antibiotic puromycin is added initially to transfected cells at a rate of 1 μg/ml to begin the selection process. The concentration is gradually raised in 0.25 μg/ml increments until a maximum of 2.0 μg/ml is reached. The cells do not produce much protein at this high puromycin concentration, but the puromycin kills any non-transfected cells. Once the non-transfected cells are killed, the concentration was decreased to 1.25 -1.5 μg/ml to resume protein production.

This vector is called TnPuro (SEQ ID NO:7) (followed by the designation of the insulator elements; in this case TnPuro MAR (SEQ ID NO:8). MAR designates Matrix Attachment Region) and has a multiple cloning site downstream of the puro gene to facilitate cloning of the

gene of interest (GOI). To determine if the orientation of the GOI with respect to the puromycin gene had an effect, two vectors were constructed in the TnPuro MAR backbone; one in which the puromycin/GOI were tail to head (poly A to promoter (SEQ ID NO:29), and the second in which the two genes were tail to tail (poly A to polyA (SEQ ID NO:28). The tail to tail version produced 3.4 times more protein than the tail to head version (52.5 μg/ml compared to 15.5 μg/ml).

Using tubular gland cell culture, each backbone vector was tested using the ovalbumin promoter/hGH as the GOI. Again, the TnMAR (SEQ ID NO:26) produced as much as 40% more protein as the backbone vector using the HS4 insulator elements and, in this experiment, it produced about 37% more protein than the CMV promoter/hGH vector using the HS4 insulators (data not shown).

In specific embodiments, the disclosed backbone vectors are defined by the following annotations:

SEQ ID NO:1 pTnMCS (base vector, without MCS extension) Vector #5001

Bp 1 – 130	Remainder of F1 (-) ori of pBluescriptll sk(-) (Stratagene) bp1-130
Bp 133 – 1812	CMV promoter/enhancer taken from vector pGWIZ (Gene Therapy Systems) bp229-1873
Bp 1813 – 3018	Transposase, modified from Tn10 (GeneBank accession #J01829) Bp 108-1316
Bp 3019 – 3021	Engineered stop codon
Bp 3022 – 3374	Non-coding DNA from vector pNK2859
Bp 3375 – 3417	Lambda DNA from pNK2859
Bp 3418 – 3487	70bp of IS10 left from Tn10
Bp 3494 – 3700	Multiple cloning site from pBluescriptII sk(-), thru the XmaI site Bp 924-718
Bp 3701 – 3744	Multiple cloning site from pBluescriptII sk(-), from the XmaI site thru the XhoI site. These base pairs are usually lost when cloning into pTnMCS. Bp 717-673
Bp 3745 – 4184	Multiple cloning site from pBluescriptII sk(-), from the XhoI site bp 672-235
Bp 4190 – 4259	70 bp of IS10 from Tn10
Bp 4260 – 4301	Lambda DNA from pNK2859
Bp 4302 – 5167	Non-coding DNA from pNK2859
Bp 5168 – 7368	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO: 2	*pTnX-MCS (Vector #5005) (pTNMCS (base vector) with MCS extension)*
Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) Bp 4-135
Bp 133 – 1785	CMV Promoter/Enhancer from vector pGWIZ (Gene Therapy Systems)
Bp 1786 – 3018	Transposase, modified from Tn10 (GeneBank accession #J01829) Bp 81-1313
Bp 3019 – 3021	Engineered stop codon
Bp 3022 – 3374	Non-coding DNA from vector pNK2859
Bp 3375 – 3416	Lambda DNA from pNK2859
Bp 3417 – 3486	70bp of IS10 left from Tn10 (GeneBank accession #J01829 Bp 1-70)
Bp 3487 – 3704	Multiple cloning site from pBluescriptII sk(-), thru XmaI
Bp 3705 – 3749	Multiple cloning site from pBluescriptII sk(-), from XmaI thru XhoI
Bp 3750 – 3845	Multiple cloning site extension from XhoI thru PspOMI
BP 3846 - 4275	Multiple cloning site from pBluescriptII sk(-), from PspOMI
Bp 4276 – 4345	70 bp of IS10 from Tn10 (GeneBank accession #J01829 Bp 70-1)
Bp 4346 – 4387	Lambda DNA from pNK2859
Bp 4388 – 5254	Non-coding DNA from pNK2859
Bp 5255 – 7455	pBluescriptII sk(-) base vector (Stratagene, INC) Bp 761-2961

SEQ ID NO: 3	*HS4 Flanked BV (Vector #5006)*
Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) Bp 4-135
Bp 133 – 1785	CMV Promoter/Enhancer from vector pGWIZ (Gene Therapy Systems) Bp 229-1873, including the combination of 2 NruI cut sites
Bp 1786 – 3018	Transposase, modified from Tn10 (GeneBank accession #J01829) Bp 81-1313
Bp 3019 – 3021	Engineered stop codon
Bp 3022 – 3374	Non-coding DNA from vector pNK2859
Bp 3375 – 3416	Lambda DNA from pNK2859
Bp 3417 – 3490	70bp of IS10 left from Tn10 (GeneBank accession #J01829 Bp 1-70)
Bp 3491 – 3680	Multiple cloning site from pBluescriptII sk(-), thru NotI Bp 926-737
Bp 3681 – 4922	HS4 – Beta-globin Insulator Element from Chicken gDNA
Bp 4923 – 5018	Multiple cloning site extension XhoI thru MluI

Bp 5019 – 6272	HS4 – Beta-globin Insulator Element from Chicken gDNA
Bp 6273 – 6342	70 bp of IS10 from Tn10 (GeneBank accession #J01829 Bp 70-1)
Bp 6343 – 6389	Lambda DNA from pNK2859
Bp 6390 – 8590	pBluescriptII sk(-) base vector (Stratagene, INC) Bp 761-2961

SEQ ID NO: 4	*pTn-10 HS4 Flanked Backbone (Vector #5012)*
Bp. 1 - 132	Remaining of F1 (-) Ori from pBluescript II sk(-) (Statagene Bp 4-135).
Bp. 133 - 1806	CMV Promoter / Enhancer from vector pGWIZ (Gene Therapy Systems) Bp. 229-1873.
Bp. 1807 - 3015	Tn-10 transposase, from pNK2859 (GeneBank accession #J01829 Bp. 81-1313).
Bp. 3016 - 3367	Non-coding DNA, possible putative poly A, from vector pNK2859.
Bp. 3368 - 3410	Lambda DNA from pNK2859.
Bp. 3411 - 3480	70bp of IS 10 left from Tn10 (GeneBank accession #J01829 bp. 1-70
Bp. 3481 - 3674	Multiple cloning site from pBluescript II sk(-), thru NotI Bp. 926-737.
Bp. 3675 - 4916	Chicken Beta Globin HS4 Insulator Element (Genbank Accession #NW_060254.0).
Bp. 4917 - 5012	Multiple cloning site extension Xho I thru Mlu I.
Bp. 5013 - 6266	Chicken Beta Globin HS4 Insulator Element (Genbank Accession #NW_060254.0).
Bp. 6267 - 6337	70bp of IS 10 left from Tn10 (GeneBank accession #J01829 bp. 1-70
Bp. 6338 - 6382	Lambda DNA from pNK2859.
Bp. 6383 - 8584	pBluescript II sk(-) Base Vector (Stratagene, Inc. Bp. 761-2961).

SEQ ID NO: 5	*pTn10 MAR Flanked BV (Vector 5018)*
Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)

Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 3016 – 3367	Putative PolyA from vector pNK2859
Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3481 – 3651	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 5367	Lysozyme Matrix Attachment Region (MAR)
Bp 5368 – 5463	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru MluI
Bp 5464 - 7168	Lysozyme Matrix Attachment Region (MAR)
Bp 7169 – 7238	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 7239 - 7281	Lambda DNA from pNK2859
Bp 7282 - 9486	pBluescriptII sk(-) base vector (Stratagene, INC)

SEQ ID NO:6 Vector 5020; pTn10 PURO – LysRep2 Flanked BV

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 3016 – 3367	Putative PolyA from vector pNK2859
Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3481 – 3484	Synthetic DNA added during construction
Bp 3485 – 3651	pBluescriptII sk(-) base vector (Stratagene, INC) bp 926-760

Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 4608	Lysozyme Rep2 from gDNA (corresponds to Genbank Accession #NW_060235)
Bp 4609 – 4686	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru BsiWI
Bp 4687 - 4999	HSV-TK polyA from pS65TC1 bp 3873-3561
Bp 5000 - 5028	Excess DNA from pMOD PURO (invivoGen)
BP 5029 - 5630	Puromycin resistance gene from pMOD PURO (invivoGen) bp 717-116
Bp 5631 - 6016	SV40 promoter from pS65TC1, bp 2232-2617
Bp 6017 - 6022	MluI RE site
Bp 6023 - 6956	Lysozyme Rep2 from gDNA (corresponds to Genbank Accession #NW_060235)
Bp 6957 – 6968	Synthetic DNA added during construction including a PspOMI RE site
Bp 6969 – 7038	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 7039 - 7081	Lambda DNA from pNK2859
Bp 7082 - 7085	Synthetic DNA added during construction
Bp 7086 - 9286	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO:7 (Vector #5019 pTN-10 PURO – HS4 Flanked BV)

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 3016 – 3367	Putative PolyA from vector pNK2859
Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)

Bp 3481 – 3484	Synthetic DNA added during construction
Bp 3485 – 3651	pBluescriptII sk(-) base vector (Stratagene, INC) bp 926-760
Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 4916	Chicken HS4-Beta Globin enhancer element from gDNA (corresponds to Genbank Accession #NW_060254 bp 215169-216410)
Bp 4917 – 4994	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru BsiWI
Bp 4995 - 5307	HSV-TK polyA from pS65TC1 bp 3873-3561
Bp 5308 - 5336	Excess DNA from pMOD PURO (invivoGen)
BP 5337 - 5938	Puromycin resistance gene from pMOD PURO (invivoGen) bp 717-116
Bp 5939 - 6324	SV40 promoter from pS65TC1, bp 2232-2617
Bp 6325 - 6330	MluI RE site
Bp 6331 - 7572	Chicken HS4-Beta Globin enhancer element from gDNA (corresponds to Genbank Accession #NW_060254 bp 215169-216410)
Bp 7573 – 7584	Synthetic DNA added during construction including a PspOMI RE site
Bp 7585 – 7654	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 7655 - 7697	Lambda DNA from pNK2859
Bp 7698 - 7701	Synthetic DNA added during construction
Bp 7702 - 9902	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO: 8	*Vector #5021; pTn10 PURO - MAR Flanked BV*
Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 3016 – 3367	Putative PolyA from vector pNK2859

Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3481 – 3651	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 5367	Lysozyme Matrix Attachment Region (MAR)
Bp 5368 – 5445	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru BsiWI
Bp 5446 - 5758	HSV-TK polyA from pS65TC1 bp 3873-3561
BP 5759 - 6389	Puromycin resistance gene from pMOD PURO (invivoGen)
Bp 6390 - 6775	SV40 promoter from pS65TC1, bp 2232-2617
Bp 6776 - 8486	Lysozyme Matrix Attachment Region (MAR)
Bp 8487 – 8556	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 8557 - 8599	Lambda DNA from pNK2859
Bp 8600 - 10804	pBluescriptII sk(-) base vector (Stratagene, INC)

SEQ ID NO:9 Vector #5022; pTn10 Gen – MAR Flanked BV

Bp 1 – 5445	pTn10 MAR Flanked BV, ID #5018
Bp 5446 - 5900	HSV-TK polyA from Taken from pIRES2-ZsGreen1, bp 4428-3974
Bp 5901 - 6695	Kanamycin/Neomycin (G418) resistance gene, taken from pIRES2-ZsGreen1, Bp 3973-3179
Bp 6696 - 7046	SV40 early promoter/enhancer taken from pIRES2-ZsGreen1,bp 3178-2828
Bp 7047 - 7219	Bacterial promoter for expression of KAN resistance gene, taken from pIRES2-ZsGreen1,bp 2827-2655
Bp 7220 - 11248	pTn10 MAR Flanked BV, bp 5458-9486

SEQ ID NO: 10	*pTn10 MAR Flanked BV; Vector #5024*
Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 154	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 155 - 229	CMV promoter (from vector pGWIZ, Gene Therapy Systems bp 844-918
Bp 230 - 350	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 351 - 1176	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)

Bp 1177 - 1184	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1185 – 1213	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1214 – 2422	Transposon, modified from Tn10 GenBank Accession #J01829 bp 108-1316
Bp 2423 – 2774	Putative PolyA from vector pNK2859
Bp 2775 – 2817	Lambda DNA from pNK2859
Bp 2818 – 2887	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 2888 – 3058	pBluescriptII sk(-) base vector (Stratagene, INC) Bp 3059 - 3081 Multiple cloning site from pBluescriptll sk(-)thru NotI,
Bp 3082 - 4774	Chicken 5' Lysozyme Matrix Attachment Region (MAR) from chicken gDNA corresponding to GenBank Accession #X98408
Bp 4775 – 4870	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru MluI
Bp 4871 - 6575	Chicken 3' Lysozyme Matrix Attachment Region (MAR) from chicken gDNA corresponding to GenBank Accession #X98408
Bp 6576 – 6645	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 6646 - 6688	Lambda DNA from pNK2859
Bp 6689 - 8893	pBluescriptII sk(-) base vector (Stratagene, INC)

SEQ ID NO: 11	*Vector #5025; pTn10 (-CMV Enh.)PURO - MAR Flanked BV*
Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 154	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 155 - 229	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 230 - 350	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 351 - 1176	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1177 - 1184	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1185 – 1213	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1214 – 2422	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 2423 – 2774	Putative PolyA from vector pNK2859
Bp 2775 – 2817	Lambda DNA from pNK2859

Bp 2818 – 2887	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 2888 – 3058	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3059 - 3081	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3082 - 4774	Lysozyme Matrix Attachment Region (MAR) from chicken gDNA corresponding to GenBank Accession #X98408
Bp 4775 – 4852	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru BsiWI
Bp 4853 - 5165	HSV-TK polyA from pS65TC1 bp 3873-3561
BP 5166 - 5796	Puromycin resistance gene from pMOD PURO (invivoGen)
Bp 5797 - 6182	SV40 promoter from pS65TC1, bp 2232-2617
Bp 6183 - 7893	Lysozyme Matrix Attachment Region (MAR)
Bp 7894 – 7963	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 7964 - 8010	Lambda DNA from pNK2859
Bp 8011 - 10211	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO: 12	*Vector #5026; pTn10 MAR Flanked BV*
Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 154	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 155 - 540	SV40 promoter from pS65TC1 bp 2232-2617
Bp 541 - 661	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 662 - 1487	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1488 - 1495	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1496 – 1524	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1525 – 2733	Transposon, modified from Tn10 GenBank Accession #J01829 bp 108-1316
Bp 2734 – 3085	Putative PolyA from vector pNK2859
Bp 3086 – 3128	Lambda DNA from pNK2859
Bp 3129 – 3198	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3199 – 3369	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3370 - 3392	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3393 - 5085	Chicken 5' Lysozyme Matrix Attachment Region (MAR) from chicken gDNA corresponding to GenBank Accession #X98408

Bp 5086 – 5181	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru MluI
Bp 5182 - 6886	Chicken 3' Lysozyme Matrix Attachment Region (MAR) from chicken gDNA corresponding to GenBank Accession #X98408
Bp 6887 – 6956	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 6957 - 6999	Lambda DNA from pNK2859
Bp 7000 - 9204	pBluescriptII sk(-) base vector (Stratagene, INC)

SEQ ID NO: 13 *pTn10 SV 40 Pr.PURO - MAR Flanked BV (Vector #5027)*

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene)bp 4-135
Bp 133 – 154	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 155 - 540	SV40 Promoter from pS65TC1, Bp 2232-2617
Bp 541 - 661	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 662 - 1487	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1488 - 1495	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1496 – 1524	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1525 – 2733	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 2734 – 3085	Putative PolyA from vector pNK2859
Bp 3086 – 3128	Lambda DNA from pNK2859
Bp 3129 – 3198	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3199 – 3369	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3370 - 3392	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3393 - 5085	Lysozyme Matrix Attachment Region (MAR) from chicken gDNA GenBank Accession #X98408.
Bp 5086 – 5163	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru BsiWI
Bp 5164 - 5476	HSV-TK polyA from pS65TC1 bp 3873-3561
BP 5477 - 6107	Puromycin resistance gene from pMOD PURO (invivoGen)
Bp 6108 - 6499	SV40 promoter from pS65TC1, bp 2232-2617
Bp 6500 - 8204	Lysozyme Matrix Attachment Region (MAR)
Bp 8205 – 8274	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 8275 - 8317	Lambda DNA from pNK2859

Bp 8318 - 10522 pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

In specific embodiments, the disclosed hybrid promoters are defined by the following annotations:

SEQ ID NO:14 (CMV/Oval promoter Version 1 = ChOvp/CMVenh/CMVp)

Bp 1 - 840: corresponds to bp 421-1260 from the chicken ovalbumin promoter, GenBank accession number

Bp 841- 1439: CMV Enhancer bp 245-843 taken from vector pGWhiz CMV promoter and enhancer bp 844-918 taken from vector pGWhiz (includes the CAAT box at 857-861 and the TATA box at 890-896).

Bp 1440-1514 CMV promoter

SEQ ID NO:15 (CMV/Oval promoter Version 2 = ChSDRE/CMVenh/ChNRE/CMVp)

Bp 1 - 180: Chicken steroid dependent response element from ovalbumin promoter

Bp 181 - 779: CMV Enhancer bp 245-843 taken from vector pGWhiz

Bp 780 - 1049: Chicken ovalbumin promoter negative response element

Bp 1050 - 1124: CMV promoter bp 844-918 taken from vector pGWhiz (includes the CAAT box at 857-861 and the TATA box at 890-896. Some references overlap the enhancer to different extents.)

In specific embodiments, the disclosed expression vectors are defined by the following annotations:

SEQ ID NO:17 (Vector # 153) HS4 Flanked Backbone Vector (CMV.Ovalp vs. 1)

Bp 1 - 4936 HFBV

Bp 4937 - 5782 Chicken Ovalbumin enhanced promoter (bp 1090-1929) w/ EcoRI site at 3' end for ligation

Bp 5783 - 6437 CMVenhancer/promoter (bp 245-899 of gWIZ blank vector)

Bp 6438 - 6459 XhoI site + bp 900-918 of CMVpromoter from gWIZ blank vector (site used to add on the CMViA')

Bp 6460 - 7420 CMV intron A' (bp 919-1873 of gWIZ; includes CMV immediate-early gene, Exon1; CMV intron A; CMV immediate-early gene, partial Exon 2) + SalI cut site for ligation

Bp 7421 - 7489 Chicken Conalbumin Signal Sequence + Kozak sequence 7421-7426 (from GenBank Accession # X02009) + BsrFI site for ligation

Bp 7490 - 7540 3x flag

Bp 7541 - 7555	Enterokinase Cleavage Site
Bp 7556 - 8137	Human Growth Hormone, for Chicken from GenBank Accession #V00520, bp 140-715 (start codon and signal sequence omitted) + AatII site at 3' end for ligation
Bp 8138 - 9054	Chicken Ovalbumin PolyA site (GenBank Accession # J00895 bp 8260-9176)
Bp 9055 - 10038	Chicken Ovalbumin PolyA Extension (GenBank Accession X01422 bp 271-1255)
Bp 10039 – 13675	HFBV (bp 4954 – 8590)

SEQ ID NO:18	*Vector #173; HS4 Flanked Backbone Vector (CMV Ovalp. vs. 2)*
Bp 1 - 4936	HS4 Flanked Backbone vector
Bp 4937 - 5122	Chicken SDRE (from ChOVep, bp 1100-1389) with EcoRI site at 3' end for ligations (bp 5117-5122)
Bp 5123 - 5727	CMVenhancer (from gWIZ blank vector, bp 245-843) with NgoMIV site at 3' end for ligations (5721-5727)
Bp 5728 - 6003	Chicken NRE (from ChOvep, bp 1640-1909) with KpnI site at 3' end for ligations (bp 5998-6003)
Bp 6004 - 6081	CMVpromoter (from gWIZ blank vector, bp 844-915); has XhoI site from vector (inserted "CTC" at bp 6060 to create XhoI site to ligate vector to CMViA').
Bp 6082 - 7042	CMV Intron A' (CMV immediate early gene, exon 1; CMV Intron A; CMV immediate early gene, partial exon 2); from gWIZ blank vector bp 919-1873, with SalI site at 3' end for ligation (bp 7037-7042)
Bp 7043 - 7111	Chicken Conalbumin Signal Sequence + Kozak sequence 7043-7048 (from GenBank Accession # X02009) + BsrFI site for ligation
Bp 7112 - 7162	3x flag
Bp 7163 - 7177	Enterokinase Cleavage Site
Bp 7178 - 7759	Human Growth Hormone, for Chicken from GenBank Accession #V00520, bp 140-715 (start codon and signal sequence omitted) + AatII site at 3' end for ligation
Bp 7760 - 8675	Chicken Ovalbumin PolyA site (GenBank Accession # J00895 bp 8260-9176)

Bp 8676 - 9660 Chicken Ovalbumin PolyA Extension (GenBank Accession # X01422 bp 271-1255)

Bp 9661 – 13297 HS4 flanked Backbone vector (bp 4954 – 8590)

SEQ ID NO:19 (Vector #174 HS4 Flanked Backbone Vector (CMV.Ovalp vs. 3)

Bp 1 - 4936 HS4 Flanked Backbone vector (bp 1-4936)

Bp 4937 - 5542 CMVenhancer (from gWIZ blank vector, bp 245-843) with NgoMIV site at 3' end

Bp 5543 - 6387 Chicken Ovalbumin promoter (contains CH SDRE, promoter, NRE) bp 1090-1929 of CHOvep; with KpnI site at the 3' end

Bp 6388 - 6465 CMVpromoter (from gWIZ blank vector, bp 844-915); has XhoI site from vector (inserted "CTC" at bp 6060 to create XhoI cut to ligate clone 10 to CMViA').

Bp 6466 - 7426 CMV Intron A' (CMV immediate early gene, exon 1; CMV Intron A; CMV immediate early gene, partial exon 2); from gWIZ blank vector bp 919-1873, with SalI site at 3' end for ligation (bp 7037-7042)

Bp 7427 - 7495 Chicken Conalbumin Signal Sequence + Kozak sequence (7427-7432) (from GenBank Accession # X02009) + BsrFI site for ligation

Bp 7496 - 7546 3x flag

Bp 7547 - 7561 Enterokinase Cleavage Site

Bp 7562 - 8143 Human Growth Hormone, for Chicken from GenBank Accession #V00520, bp 140-715 (start codon and signal sequence omitted) + AatII site at 3' end for ligation

Bp 8144 - 9059 Chicken Ovalbumin PolyA site (GenBank Accession # J00895 bp 8260-9176)

Bp 9060 – 10044 Chicken Ovalbumin PolyA Extension (GenBank Accession #X01422 bp 271-1255)

Bp 10045 – 13681 HS4 flanked Backbone vector (bp 4954 – 8590)

SEQ ID NO:20 (# 157) HS4 Flanked Backbone with OV enh/prom./OV ia/ OVg' FL/ nrs/ 3XF/ co-hGH/ OV ext.PA)

Bp 1 - 132 Remaining of F1 (-) Ori from pBluescript II sk(-)Statagene Bp 4-135).

Bp 133 - 1812 CMV Promoter / Enhancer from vector pGWIZ (Gene Therapy Systems) Bp. 229-1873.

Bp 1813 - 3021	Tn-10 transposase, from pNK2859 (GeneBank accession #J01829 Bp. 81-1313).
Bp 3022 - 3373	Non-coding DNA, possible putative poly A, from vector pNK2859.
Bp 3374 - 3416	Lambda DNA from pNK2859.
Bp 3417 - 3486	70bp of IS 10 left from Tn10 (GeneBank accession #J01829 bp. 1-70
Bp 3487 - 3680	Multiple cloning site from pBluescript II sk(-), thru NotI Bp. 926-737.
Bp 3681 - 4922	Chicken Beta Globin HS4 Insulator Element (Genbank accession #NW_060254.0).
Bp 4923 - 4931	Multiple cloning site extension Xho I thru Asc I.
Bp 4932 - 5600	Chicken Ovalbumin Enhancer GenBank Accession Number J00895.
Bp 5601 - 6950	Chicken Ovalbumin Promoter GenBank accession #J00895 and M24999
Bp 6951 - 9756	Chicken Ovalbumin full length gene with Intron A Included. (Gene Bank accession # J00895). Kozak (8598-8603).
Bp 9757 - 9817	Synthetically produced New Rotational Spacer.
Bp 9818 - 9883	3xFlag with Enterokinase Cleavage Site
Bp 9884 - 10461	human Growth Hormone for Chicken (Genbank accession #V00520 bp. 140-715, Start codon and signal sequence omitted)
Bp 10462 - 12362	Chicken Ovalbumin Extended Poly A(genbank accession # J00895 and X01422.)
Bp 12363 - 12422	Multiple cloning site extension Pac I thru Mlu I.
Bp 12423 - 13676	Chicken Beta Globin HS4 Insulator Element (Genbank accession #NW_060254.0).
Bp 13677 - 13747	70bp of IS 10 left from Tn10 (GeneBank accession #J01829 bp. 1-70
Bp 13748 - 13793	Lambda DNA from pNK2859.
Bp 13794 - 15994	pBluescript II sk(-) Base Vector (Stratagene, Inc. Bp. 761-2961).

SEQ ID NO:21 ((# 181) pTn-10 HS4 Flanked Backbone with OV enh/prom./OV ia/ OVg' FL/ nrs/ 3XF/ co-hGH/ OV ext.PA)

Bp 1 - 132	Remaining of F1 (-) Ori from pBluescript II sk(-) (Statagene Bp 4-135).
Bp 133 - 1806	CMV Promoter / Enhancer from vector pGW1Z (Gene Therapy Systems) Bp. 229-1873.
Bp 1807 - 3015	Tn-10 transposase, from pNK2859 (GeneBank accession #J01829 Bp. 81-1313).
Bp 3016 - 3367	Non-coding DNA, possible putative poly A, from vector pNK2859.
Bp 3368 - 3410	Lambda DNA from pNK2859.

Bp 3411 - 3480	70bp of IS 10 left from Tn10 (GeneBank accession #J01829 bp. 1-70
Bp 3481 - 3674	Multiple cloning site from pBluescript II sk(-), thru NotI Bp. 926-737.
Bp 3675 - 4916	Chicken Beta Globin HS4 Insulator Element (Genbank accession #NW_060254.0).
Bp 4917 - 4924	Multiple cloning site extension Xho I thru Asc I.
Bp 4925 - 5594	Chicken Ovalbumin Enhancer GenBank Accession NumberJ00895.
Bp 5595 - 6944	Chicken Ovalbumin Promoter GenBank accession #J00895 and M24999
Bp 6945 - 9751	Chicken Ovalbumin full length gene with Intron A Included. (Gene Bank accession # J00895). Kozak sequence (Bp 8592-8597)
Bp 9752 - 9811	Synthetically produced New Rotational Spacer.
Bp 9812 - 9877	3xFlag with Enterokinase Cleavage Site
Bp 9878 - 10458	human Growth Hormone for Chicken (Genbank accession #V00520 bp. 140-715, Start codon and signal sequence omitted)
Bp 10459 - 12356	Chicken Ovalbumin Extended Poly A(genbank accession # J00895 and X01422.)
Bp 12357 - 12416	Multiple cloning site extension Pac I thru Mlu I.
Bp 12417 - 13671	Chicken Beta Globin HS4 Insulator Element (Genbank accession #NW_060254.0).
Bp 13672 - 13741	70bp of IS 10 left from Tn10 (GeneBank accession #J01829 bp. 1-70
Bp 13742 - 13786	Lambda DNA from pNK2859.
Bp 13787 - 15988	pBluescript II sk(-) Base Vector (Stratagene, Inc. Bp. 761-2961).

SEQ ID NO:22 ((VID # 170) pTn-10 HS4 Flanked Backbone with OV ehn/prom./OV ia/ OVg' FL/ nrs/ 3XF/ co-hGH/ OPA)

Bp 1 – 132	Remaining of F1 (-) Ori from pBluescript II sk(-) (Statagene Bp 4-135).
Bp 133 - 1806	CMV Promoter / Enhancer from vector pGWIZ (Gene Therapy Systems) Bp. 229-1873.
Bp 1807 - 3015	Tn-10 transposase, from pNK2859 (GeneBank accession #J01829 Bp. 81-1313).
Bp 3016 - 3367	Non-coding DNA, possible putative poly A, from vector pNK2859.
Bp 3368 - 3410	Lambda DNA from pNK2859.
Bp 3411 - 3480	70bp of IS 10 left from Tn10 (GeneBank accession #J01829 bp. 1-70
Bp 3481 - 3674	Multiple cloning site from pBluescript II sk(-), thru NotI Bp. 926-737.
Bp 3675 - 4916	Chicken Beta Globin HS4 Insulator Element (Genbank accession #NW_060254.0).

Bp 4917 - 4924	Multiple cloning site extension Xho I thru Asc I.
Bp 4925 - 5594	Chicken Ovalbumin Enhancer GenBank Accession Number J00895.
Bp 5595 - 6944	Chicken Ovalbumin Promoter GenBank accession #J00895 and M24999
Bp 6945 - 9751	Chicken Ovalbumin full length gene with Intron A Included. (Gene Bank accession # J00895). Kozak sequence (Bp 8592-8597)
Bp 9752 - 9811	Synthetically produced New Rotational Spacer.
Bp 9812 - 9877	3xFlag with Enterokinase Cleavage Site
Bp 9878 - 10458	human Growth Hormone for Chicken (Genbank accession #V00520 bp. 140-715, Start codon and signal sequence omitted)
Bp 10459 - 11387	Chicken Ovalbumin Regular length Poly A(genbank accession # J00895 and X01422.)
Bp 11388 – 11447	Multiple cloning site extension Pac I thru Mlu I.
Bp 11448 - 12701	Chicken Beta Globin HS4 Insulator Element (Genbank accession #NW_060254.0).
Bp 12702 - 12772	70bp of IS 10 left from Tn10 (GeneBank accession #J01829 bp. 1-70
Bp 12773 - 12817	Lambda DNA from pNK2859.
Bp 12818 - 15019	pBluescript II sk(-) Base Vector (Stratagene, Inc. Bp. 761-2961).

SEQ ID NO:23	*(# 159; Ovep-hGH-OVexpA – Cl.10 cass in HS4 Flanked BV)*
Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 - 1785	Synthetic DNA added during construction (combination of two NruI RE sites)
Bp 1786 – 3021	Transposase Tn10 GenBank Accession #J01829 Bp 81-1316
Bp 3022 – 3373	Non-coding DNA from vector pNK2859
Bp 3374 – 3416	Lambda DNA from pNK2859
Bp 3417 – 3486	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)

Bp 3487 – 3490	Synthetic DNA added during construction
Bp 3491 – 3657	pBluescriptII sk(-) base vector (Stratagene, INC) bp 926-760
Bp 3658 - 3680	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3681 - 4922	Chicken HS4-Beta Globin enhancer element from genomic (g)DNA (corresponds to Genbank Accession #NW_060254 bp 215169-216410)
Bp 4923 – 4936	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp 4937 - 5600	Chicken Ovalbumin Enhancer from gDNA (corresponds to Genbank Accession #AH003855 bp 13-675)
Bp 5601 - 6942	Chicken Ovalbummin Promoter from gDNA (corresponds to Genbank Accession #J00895 bp 1-1337)
Bp 6943 - 6948	XhoI RE site
Bp 6949 - 6964	pGWIZ base vector (Gene Therapy Systems) bp 903-918
Bp 6965 - 7085	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
BP 7086 - 7911	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 7912 - 7919	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 7920 - 7928	SalI RE site and Conalbumin Kozak sequence (Bp 7926-7931)
Bp 7929 - 7988	Conalbumin Signal Peptide (corresponds to Genbank Accession #Y00407 bp 343-385, 1699-1715)
Bp 7989 - 7994	Synthetic DNA added during construction (destroyed NgOMIV RE site)
Bp 7995 - 8045	3xFlag – no rotational spacer
Bp 8046 - 8060	Enterokinase cleavage site
Bp 8061 - 8636	Human Growth Hormone from GenBank Accession #V00520, bp 140-715 (start codon and ss omitted)
Bp 8637 - 8642	AatII RE site
Bp 8643 – 10533	Chicken Ovalbumin Extended PolyA from gDNA
Bp 10534 – 10598	MCS extension from pTN-MCS, PacI thru MluI
Bp 10599 - 11840	Chicken HS4-Beta Globin enhancer element from gDNA (corresponds to Genbank Accession #NW_060254 bp 215169-216410)
Bp 11841 – 11852	Synthetic DNA added during construction including a PspOMI RE site
Bp 11853 - 11922	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 11923 - 11965	Lambda DNA from pNK2859

Bp 11966 - 11969	Synthetic DNA added during construction
Bp 11970 - 14170	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO:24 *(# 202 – Ovep-hGH-OVexpA – Cl.10 cass in pTn-10 HS4 Flanked BV)*

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 3016 – 3367	Putative PolyA from vector pNK2859
Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3481 – 3484	Synthetic DNA added during construction
Bp 3485 – 3651	pBluescriptII sk(-) base vector (Stratagene, INC) bp 926-760
Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 4916	Chicken HS4-Beta Globin enhancer element from gDNA (corresponds to Genbank Accession #NW_060254 bp 215169-216410)
Bp 4917 – 4930	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp 4931 - 5593	Chicken Ovalbumin Enhancer from gDNA (corresponds to Genbank Accession #AH003855 bp 13-675)
Bp 5594 - 5599	HindIII RE site
BP 5600 - 6936	Chicken Ovalbummin Promoter from gDNA (corresponds to Genbank Accession #J00895 bp 1-1337)
Bp 6937 - 6942	XhoI RE site
Bp 6943 - 6958	pGWIZ base vector (Gene Therapy Systems) bp 903-918
Bp 6959 - 7079	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)

BP 7080 - 7905	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 7906 - 7913	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 7914 - 7922	SalI RE site and Conalbumin Kozak sequence (Bp 7920-7925)
Bp 7923 - 7982	Conalbumin Signal Peptide (corresponds to Genbank Accession #Y00407 bp 343-385, 1699-1715)
Bp 7983 - 7988	Synthetic DNA added during construction (destroyed NgOMIV RE site)
Bp 7989 - 8039	3xFlag – no rotational spacer
Bp 8040 - 8054	Enterokinase cleavage site
Bp 8055 - 8630	Human Growth Hormone from GenBank Accession #V00520, bp 140-715 (start codon and ss omitted)
Bp 8631 - 8636	AatII RE site
Bp 8637 – 9568	Chicken Ovalbumin 3'Exon 7 and polyA from gDNA (corresponds to GenBank Accession #J00829 bp 8260-9191)
Bp 9569 – 10527	Chicken Ovalbumin 3'terminus from gDNA (corresponds to GenBank Accession #X01422 bp 286-1244)
Bp 10528 – 10531	Synthetic DNA added during construction (destroyed PacI RE site)
Bp 10532 – 10588	MCS extension from pTN-MCS, KasI thru MluI
Bp 10589 - 11830	Chicken HS4-Beta Globin enhancer element from gDNA (corresponds to Genbank Accession #NW_060254 bp 215169-216410)
Bp 11831 – 11842	Synthetic DNA added during construction including a PspOMI RE site
Bp 11843 - 11912	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 11913 - 11955	Lambda DNA from pNK2859
Bp 11956 - 11959	Synthetic DNA added during construction
Bp 11960 - 14160	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO:25 (#203 - Ovep-hGH-OVexpA – Cl.10 cass in pTn-10 LysRep2 Flanked BV)

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)

Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 3016 – 3367	Putative PolyA from vector pNK2859
Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3481 – 3484	Synthetic DNA added during construction
Bp 3485 – 3651	pBluescriptII sk(-) base vector (Stratagene, INC) bp 926-760
Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 4608	Lysozyme Rep2 from gDNA (corresponds to Genbank Accession #NW_060235)
Bp 4609 – 4622	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp 4623 - 5285	Chicken Ovalbumin Enhancer from gDNA (corresponds to Genbank Accession #AH003855 bp 13-675)
Bp 5286 - 5291	HindIII RE site
BP 5292 - 6628	Chicken Ovalbummin Promoter from gDNA (corresponds to Genbank Accession #J00895 bp 1-1337)
Bp 6629 - 6634	XhoI RE site
Bp 6635 - 6650	pGWIZ base vector (Gene Therapy Systems) bp 903-918
Bp 6651 - 6771	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
BP 6772 - 7597	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 7598 - 7605	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 7606 - 7614	SalI RE site and Conalbumin Kozak sequence (Bp 7612-7617)
Bp 7615 - 7674	Conalbumin Signal Peptide (corresponds to Genbank Accession #Y00407 bp 343-385, 1699-1715)
Bp 7675 - 7680	Synthetic DNA added during construction (destroyed NgOMIV RE site)
Bp 7681 - 7731	3xFlag – no rotational spacer
Bp 7732 - 7746	Enterokinase cleavage site

Bp 7747 - 8322	Human Growth Hormone from GenBank Accession #V00520, bp 140-715 (start codon and signal sequence omitted)
Bp 8323 - 8328	AatII RE site
Bp 8329 – 9260	Chicken Ovalbumin 3'Exon 7 and polyA from gDNA (corresponds to GenBank Accession #J00829 bp 8260-9191)
Bp 9261 – 10219	Chicken Ovalbumin 3'terminus from gDNA (corresponds to GenBank Accession #X01422 bp 286-1244)
Bp 10220 – 10266	MCS extension from pTN-MCS, PacI thru MluI
Bp 10267 - 11200	Lysozyme Rep2 from gDNA (corresponds to Genbank Accession #NW_060235)
Bp 11201 – 11212	Synthetic DNA added during construction including a PspOMI RE site
Bp 11213 - 11282	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 11283 - 11325	Lambda DNA from pNK2859
Bp 11326 - 11329	Synthetic DNA added during construction
Bp 11330 - 13530	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO:26 (ID# 204 - Ovep-hGH-OVexpA – Cl.10 cass in pTn-10 MAR Flanked BV)

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 3016 – 3367	Putative PolyA from vector pNK2859
Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3481 – 3484	Synthetic DNA added during construction
Bp 3485 – 3651	pBluescriptII sk(-) base vector (Stratagene, INC) bp 926-760

Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 5367	Chicken Lysozyme Matrix Attachment region (MAR) from gDNA
Bp 5368 – 5381	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp 5382 - 6044	Chicken Ovalbumin Enhancer from gDNA (corresponds to Genbank Accession #AH003855 bp 13-675)
Bp 6045 - 6050	HindIII RE site
BP 6051 - 7387	Chicken Ovalbummin Promoter from gDNA (corresponds to Genbank Accession #J00895 bp 1-1337)
Bp 7388 - 7393	XhoI RE site
Bp 7394 - 7409	pGWIZ base vector (Gene Therapy Systems) bp 903-918
Bp 7410 - 7530	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
BP 7531 - 8356	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 8357 - 8364	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 8365 - 8373	SalI RE site and Conalbumin Kozak sequence (Bp 8371-8376)
Bp 8374 - 8433	Conalbumin Signal Peptide (corresponds to Genbank Accession #Y00407 bp 343-385, 1699-1715)
Bp 8434 - 8439	Synthetic DNA added during construction (destroyed NgOMIV RE site)
Bp 8440 - 8490	3xFlag – no rotational spacer
Bp 8491 - 8505	Enterokinase cleavage site
Bp 8506 - 9081	Human Growth Hormone from GenBank Accession #V00520, bp 140-715 (start codon and ss omitted)
Bp 9082 - 9087	AatII RE site
Bp 9088 – 10019	Chicken Ovalbumin 3'Exon 7 and polyA from gDNA (corresponds to GenBank Accession #J00829 bp 8260-9191)
Bp 10020 – 10978	Chicken Ovalbumin 3'terminus from gDNA (corresponds to GenBank Accession #X01422 bp 286-1244)
Bp 10979 – 10982	Synthetic DNA added during construction (destroyed PacI RE site)
Bp 10983 – 11039	MCS extension from pTN-MCS, KasI thru MluI
Bp 11040 - 12732	Chicken Lysozyme Matrix Attachment region (MAR) from gDNA
Bp 12733 – 12744	Synthetic DNA added during construction including a PspOMI RE site
Bp 12745 - 12814	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)

Bp 12815 - 12857	Lambda DNA from pNK2859
Bp 12858 - 12861	Synthetic DNA added during construction
Bp 12862 - 15062	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO:27 (#171 – HS4(CMVpr-iA-CONss-3xFlag-hGH.CO-synPA)HS4)

Bp 1 – 4922	HS4 flanked MCS backbone vector
Bp 4923 – 4928	Restriction enzyme site, XhoI
Bp 4929 – 5542	CMV enhancer taken from pGWIZ (Gene therapy systems), bp 230-843.
Bp 5543 – 5617	CMV promoter taken from pGWIZ (Gene therapy systems), bp 844-918.
Bp 5618 – 5738	CMV Immediate early gene, Exon 1 taken from pGWIZ (Gene therapy systems), bp 919-1039.
Bp 5739 – 6564	CMV intron A taken from pGWIZ (Gene therapy systems), bp 1040-1865.
Bp 6565 – 6572	CMV Immediate early gene, partial Exon 2 taken from pGWIZ (Gene therapy systems), bp 1866-1873.
Bp 6573 – 6578	Restriction enzyme site, SalI
Bp 6579 – 6641	Conalbumin signal peptide, Corresponds to GenBank Accession Number Y00407 Bp 340-385,1699-1715. Kozak sequence (Bp 6579-6584)
Bp 6642 – 6713	3xFlag. Synthetic sequence consisting of a purification tag and enterokinase cleavage site.
Bp 6714 – 7289	Human Growth hormone for chicken, using GeneBank Accession Number V00520, bp 140-715(start codon and signal sequence omitted).
Bp 7290 – 7723	Poly A taken from vector pGWIZ (Gene Therapy Systems), bp 1900-2334.
Bp 7724 – 7729	Restriction enzyme site, XhoI
Bp 7730 – 11391	HS4 flanked MCS backbone vector BP 4929-8590

SEQ ID NO:28 (#217 – 11F TnPuro-MAR FBV(CMVpr-iA-hGH.CO-synPA)

Bp 1 - 5367	MAR Flanked Puromycin backbone vector.
Bp 5368 - 5373	Restriction enzyme site, XhoI
Bp 5374 - 5987	CMV enhancer taken from pGWIZ (Gene therapy systems), bp 230-843.
Bp 5988 - 6062	CMV promoter taken from pGWIZ (Gene therapy systems), bp 844-918.
Bp 6063 - 6183	CMV Immediate early gene, Exon 1 taken from pGWIZ (Gene therapy systems), bp 919-1039.
Bp 6184 - 7009	CMV intron A. Taken from pGWIZ (Gene therapy systems), bp 1040-1865.

Bp 7010 - 7017	CMV Immediate early gene, partial Exon 2 taken from pGWIZ (Gene therapy systems), bp 1866-1873.
Bp 7018 - 7023	Restriction enzyme site, SalI
Bp 7024 - 7086	Conalbumin signal peptide, Corresponds to GenBank accession Number Y00407, bp 340-385,1699-1715. Kozak sequence (Bp 7024-7029)
Bp 7087 - 7158	3xFlag. Synthetic sequence consisting of a purification tag and enterokinase cleavage site.
Bp 7159 - 7734	Human Growth hormone for chicken, using GenBank Accession Number V00520, bp 140-715(start codon and signal sequence omitted).
Bp 7735 - 8168	Poly A taken from vector pGWIZ (Gene Therapy Systems), bp 1900-2334.
Bp 8169 - 8174	Restriction enzyme site, XhoI
Bp 8175 – 13605	Mar flanked Puromycin backbone vector bp 5374-10804.

SEQ ID NO:29 (240 MAR FBV(synPA-hGH.CO-iA-CMVpr)

Bp 1 - 5367	MAR Flanked Puromycin backbone vector.
Bp 5368 - 5373	Restriction enzyme site, XhoI
Bp 5374 - 5807	Poly A taken from vector pGWIZ (Gene Therapy Systems), bp 2334-1900.
Bp 5808 - 6383	Human Growth hormone for chicken, using GenBank Accession Number V00520, bp 715-140(start codon and signal sequence omitted).
Bp 6384 – 6455	3xFlag. Synthetic sequence consisting of a purification tag and enterokinase cleavage site.
Bp 6456 - 6518	Conalbumin signal peptide, Corresponds to GenBank accession Number Y00407, bp 1715-1699, 385-340. Kozak sequence (Bp 6513-6518)
Bp 6519 - 6524	Restriction enzyme site, SalI
Bp 6525 - 6532	CMV Immediate early gene, partial Exon 2 taken from pGWIZ (Gene therapy systems), bp 1873-1866.
Bp 6533 - 7358	CMV intron A. Taken from pGWIZ (Gene therapy systems), bp 1865-1040.
Bp 7359 - 7479	CMV Immediate early gene, Exon 1 taken from pGWIZ (Gene therapy systems), bp 1039-919.
Bp 7480 - 7554	CMV promoter taken from pGWIZ (Gene therapy systems), bp 918-844.
Bp 7555 - 8168	CMV enhancer taken from pGWIZ (Gene therapy systems), bp 843-230.
Bp 8169 - 8174	Restriction enzyme site, XhoI
Bp 8175 – 13605	MAR flanked Puromycin backbone vector bp 5374-10804.

SEQ ID NO:30 ID# 133 and #159 - Ovep-hGH-OVexpA – Cl.10 cass in HS4 Flanked BV

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 - 1785	Synthetic DNA added during construction (combination of two NruI RE sites)
Bp 1786 – 3021	Transposase modified from Tn10 GenBank Accession #J01829 Bp 81-1316
Bp 3022 – 3373	Non-coding DNA from vector pNK2859
Bp 3374 – 3416	Lambda DNA from pNK2859
Bp 3417 – 3486	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3487 – 3490	Synthetic DNA added during construction
Bp 3491 – 3657	pBluescriptII sk(-) base vector (Stratagene, INC) bp 926-760
Bp 3658 - 3680	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3681 - 4922	Chicken HS4-Beta Globin enhancer element from gDNA (corresponds to Genbank Accession #NW_060254 bp 215169-216410)
Bp 4923 – 4936	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp 4937 - 5600	Chicken Ovalbumin Enhancer from gDNA (corresponds to Genbank Accession #AH003855 bp 13-675)
Bp 5601 - 6942	Chicken Ovalbummin Promoter from gDNA (corresponds to Genbank Accession #J00895 bp 1-1337)
Bp 6943 - 6948	XhoI RE site
Bp 6949 - 6964	pGWIZ base vector (Gene Therapy Systems) bp 903-918
Bp 6965 - 7085	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
BP 7086 - 7911	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 7912 - 7919	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)

Bp 7920 - 7928	SalI RE site and Conalbumin Kozak sequence (Bp 7926-7931)
Bp 7929 - 7988	Conalbumin Signal Peptide (corresponds to Genbank Accession #Y00407 bp 343-385, 1699-1715)
Bp 7989 - 7994	Synthetic DNA added during construction (destroyed NgOMIV RE site)
Bp 7995 - 8045	3xFlag – no rotational spacer
Bp 8046 - 8060	Enterokinase cleavage site
Bp 8061 - 8636	Human Growth Hormone from V00520, bp 140-715 (start codon and ss omitted)
Bp 8637 - 8642	AatII RE site
Bp 8643 – 10533	Chicken Ovalbumin Extended PolyA from gDNA
Bp 10534 – 10598	MCS extension from pTN-MCS, PacI thru MluI
Bp 10599 - 11840	Chicken HS4-Beta Globin enhancer element from gDNA (corresponds to Genbank Accession #NW_060254 bp 215169-216410)
Bp 11841 – 11852	Synthetic DNA added during construction including a PspOMI RE site
Bp 11853 - 11922	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 11923 - 11965	Lambda DNA from pNK2859
Bp 11966 - 11969	Synthetic DNA added during construction
Bp 11970 - 14170	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO: 31 Vector # 230 - CMV-Ovalp Vs.1 - hGH-CCG - OVexpA – in pTn-10 PURO-MAR Flanked BV

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316

Bp 3016 – 3367	Putative PolyA from vector pNK2859
Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3481 – 365I	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 5367	Chicken Lysozyme Matrix Attachment region (MAR) from gDNA
Bp 5368 – 5381	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp 5382 - 6223	Chicken Ovalbumin promoter from gDNA (Genbank Accession #J00895 bp 421-1261)
BP 6224 - 6827	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)with 5' EcoRI RE site
Bp 6828 - 6905	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-899, CTC, 900-918)
Bp 6906 - 7026	CMV Immediate Early Gene, Exon I (vector pGWIZ, Gene Therapy Systems bp 919-1039)
BP 7027 - 7852	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 7853 - 7860	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 7861 - 7935	Conalbumin Signal Peptide (Genbank #Y00407) with 5'SalI RE site. Kozak sequence (Bp 7867-7872)
Bp 7936 - 7986	3xFlag – no rotational spacer
Bp 7987 - 8001	Enterokinase cleavage site
Bp 8002 - 8577	Human Growth Hormone-codon optimized (with CCG correction) GenBank #V00520 bp I40-715
Bp 8578 - 9515	Chicken Ovalbumin 3'Exon 7 and polyA (GenBank #J00895 bp 8260-9191) with 5'AatII RE site
Bp 95I6 – 10484	Chicken Ovalbumin 3'terminus (GenBank #X01422 bp 288-1256)
Bp 10485 – 10531	MCS extension from pTN-MCS, PacI thru BsiWI
Bp 10532 – 10844	HSV-TK polyA from pS65TC1 bp 3873-3561
BP 10845 - 11475	Puromycin resistance gene from pMOD PURO (invivoGen)
Bp 11476 - 11867	SV40 promoter from pS65TC1, bp 2617-2232 with 5' MluI RE site
Bp 11868 - 13572	Chicken Lysozyme Matrix Attachment region (MAR) from gDNA
Bp 13573 - 13642	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)

Bp 13643 - 13685	Lambda DNA from pNK2859
Bp 13686 - 15890	pBluescriptII sk(-) base vector (Stratagene, INC)

SEQ ID NO:32 VECTOR # 5021-235

pTn-10 PURO - MAR Flanked BV (CMV.Ovalp Vs.1 cl.10-CCG OPA)

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 3016 – 3367	Putative PolyA from vector pNK2859
Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3481 – 3651	pBluescriptII sk(-) base vector (Stratagene, INC) bp 926-760
Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 5367	Lysozyme Matrix Attachment Region (MAR)
Bp 5368 – 5376	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp 5377 - 6228	Chicken Ovalbumin Promoter GenBank accession (#J00895 and M24999)
Bp 6229 - 6827	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 6828 - 6905	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 6906 - 7026	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039
Bp 7027 - 7852	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 7853 - 7860	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ, Gene Therapy Systems)bp1866-1873)
Bp 7867 - 7929	Conalbumin Signal Sequence including Kozak sequence (Bp 7867-7872) (GenBank Accession # Y00407)

Bp 7936 - 7986	3xFlag without a Rotational Spacer (Sigma)
Bp 7987 - 8001	Enterokinase Cleavage Site
Bp 8002 - 8577	Codon Optimized human Growth Hormone (GenBank Accession # V00520 bp. 140-715)
Bp 8584 - 9499	Chicken Ovalbumin Regular length Poly A(Genbank accession # J00895 and X01422.)
Bp 9500 - 9546	Cloning Site Extension from pTn X-MCS, Pac I thru Bsi WI.
Bp 9547 - 9859	HSV-TK polyA from pS65TC1 bp 3873-3561
BP 9860 - 10490	Puromycin resistance gene from pMOD PURO (invivoGen)
Bp 10491 - 10876	SV40 promoter from pS65TC1, bp 2232-2617
Bp 10877 - 12587	Lysozyme Matrix Attachment Region (MAR)
Bp 12588 – 12657	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 12658 - 12700	Lambda DNA from pNK2859
Bp 12701 - 14905	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO: 33 Vector # 284-5024

pTn-10 MAR Flanked BV (Hybrid Prom. Vs. 1/ CMV ia/ Con ss/ 3XF (-rs)/ hGH-co/ OPA)

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 154	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 155 - 229	CMV promoter (from vector pGWIZ, Gene Therapy Systems bp 844-918
Bp 230 - 350	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 351 - 1176	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1177 - 1184	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1185 – 1213	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1214 – 2422	Transposon, modified from Tn10 GenBank Accession #J01829 bp 108-1316
Bp 2423 – 2774	Putative PolyA from vector pNK2859
Bp 2775 – 2817	Lambda DNA from pNK2859
Bp 2818 – 2887	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 2888 – 3058	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3059 - 3081	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737

Bp 3082 - 4774	Chicken 5' Lysozyme Matrix Attachment Region (MAR) from chicken gDNA corresponding to GenBank Accession #X98408
Bp 4775 – 4788	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru Asc I
Bp 4789 - 5635	Chicken Ovalbumin Promoter GenBank accession (#J00895 and M24999)
Bp 5636 - 6234	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 6235 - 6312	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 6313 - 6433	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039
Bp 6434 - 7259	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 7260 - 7273	CMV Immediate Early Gene, Partial Exon 2 and Sal 1 Restriction Enzyme site from pGWIZ Multiple cloning site.(pGWIZ, Gene Therapy Systems)bp1866-1879)
Bp 7274 - 7336	Conalbumin Signal Sequence including Kozak sequence (Bp 7274-7279) (GenBank Accession # Y00407)
Bp 7337 - 7393	3xFlag without a Rotational Spacer (Sigma)
Bp 7394 - 7408	Enterokinase Cleavage Site
Bp 7409 - 7984	Codon Optimized human Growth Hormone (GenBank Accession # V00520 bp. 140-715)
Bp 7985 - 8906	Chicken Ovalbumin Regular length Poly A(Genbank accession # J00895 and X01422.)
Bp 8907 – 8971	Multiple Cloning Site Extension from pTn X-MCS, PacI thru Mlu I
Bp 8972 - 10676	Chicken 3' Lysozyme Matrix Attachment Region (MAR) from chicken gDNA corresponding to GenBank Accession #X98408
Bp 10677 – 10746	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 10747 - 10789	Lambda DNA from pNK2859
Bp 10790 - 12994	pBluescriptII sk(-) base vector (Stratagene, INC)

SEQ ID NO: 34 Vector #285-5026

SV40 ATS MAR FBV (Hybrid Prom Vs. 1/ CMV ia/ Con ss/ 3XF/ co-hGH/ OPA

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 154	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 155 - 539	SV40 promoter from pS65TC1 bp 2232-2617
Bp 541 - 661	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)

Bp 662 - 1487	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1488 - 1495	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1496 – 1524	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1525 – 2733	Transposon, modified from Tn10 GenBank Accession #J01829 bp 108-1316
Bp 2734 – 3085	Putative PolyA from vector pNK2859
Bp 3086 – 3128	Lambda DNA from pNK2859
Bp 3129 – 3198	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3199 – 3369	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3370 - 3392	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3393 - 5085	Chicken 5' Lysozyme Matrix Attachment Region (MAR) from chicken gDNA corresponding to GenBank Accession #X98408
Bp 5086 – 5099	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp 5100 - 5946	Chicken Ovalbumin Promoter GenBank accession (#J00895 and M24999)
Bp 5947 - 6545	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 6546 - 6623	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 6624 - 6744	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039
Bp 6745 - 7570	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 7571 - 7584	CMV Immediate Early Gene, Partial Exon 2 and Sal I Restriction Enzyme site from pGWIZ Multiple cloning site.(pGWIZ, Gene Therapy Systems)bp1866-1879)
Bp 7585 - 7647	Conalbumin Signal Sequence including Kozak sequence (Bp 7591-7596) (GenBank Accession # Y00407)
Bp 7648 - 7704	3xFlag without a Rotational Spacer (Sigma)
Bp 7705 - 7719	Enterokinase Cleavage Site
Bp 7720 - 8295	Codon Optimized human Growth Hormone (GenBank Accession # V00520 bp. 140-715)
Bp 8296 - 9217	Chicken Ovalbumin Regular length Poly A(Genbank accession # J00895 and X01422.)
Bp 9218 - 9282	Cloning Site Extension from pTn X-MCS, Pac I thru Bsi WI.
Bp 9283 - 10987	Chicken 3' Lysozyme Matrix Attachment Region (MAR) from chicken gDNA Corresponding to GenBank Accession #X98408

Bp	10988 – 11057	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp	11058 - 11100	Lambda DNA from pNK2859
Bp	11101 - 13305	pBluescriptII sk(-) base vector (Stratagene, INC)

SEQ ID NO:35 Vector #301-5025

pTn-10 (-CMV Enh.)PURO - MAR Flanked BV (CMV.Ovalp Vs.1 cl.10-CCG OPA)

Bp	1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-)(Stratagene) bp 4-135
Bp	133 – 154	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp	155 - 229	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp	230 - 350	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp	351 - 1176	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp	1177 - 1184	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp	1185 – 1213	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp	1214 – 2422	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316
Bp	2423 – 2774	Putative PolyA from vector pNK2859
Bp	2775 – 2817	Lambda DNA from pNK2859
Bp	2818 – 2887	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp	2888 – 3058	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp	3059 - 3081	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp	3082 - 4774	Lysozyme Matrix Attachment Region (MAR) from chicken gDNA corresponding to GenBank Accession #X98408
Bp	4775 – 4788	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp	4789 - 5627	Chicken Ovalbumin Promoter GenBank accession (#J00895 and M24999)
Bp	5628 - 6234	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp	6235 - 6312	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp	6313 - 6433	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039
Bp	6434 - 7259	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp	7260 - 7267	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ, Gene Therapy Systems)bp1866-1873)

Bp 7268 - 7336	Conalbumin Signal Sequence including Kozak sequence (Bp 7274-7279) (GenBank Accession # Y00407)
Bp 7337 - 7393	3xFlag without a Rotational Spacer (Sigma)
Bp 7394 - 7408	Enterokinase Cleavage Site
Bp 7409 - 7984	Codon Optimized human Growth Hormone (GenBank Accession # V00520 bp. 140-715)
Bp 7985 - 8906	Chicken Ovalbumin Regular length Poly A(Genbank accession # J00895 and X01422.)
Bp 8907 - 8953	Cloning Site Extension from pTn X-MCS, Pac I thru Bsi WI.
Bp 8954 - 9266	HSV-TK polyA from pS65TC1 bp 3873-3561
BP 9267 - 9897	Puromycin resistance gene from pMOD PURO (invivoGen)
Bp 9898 - 10283	SV40 promoter from pS65TC1, bp 2232-2617
Bp 10284 - 11982	Lysozyme Matrix Attachment Region (MAR)
Bp 11983 – 12064	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 12065 - 12111	Lambda DNA from pNK2859
Bp 12112 - 14312	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO:36 #302-5027 pTN-10 SV 40 Pr.PURO - MAR Flanked BV (CMV.Ovalp Vs.1 cl.10-CCG OPA)

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-)(Stratagene) bp 4-135
Bp 133 – 154	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 155 - 540	SV40 Promoter from pS65TC1, Bp 2232-2617
Bp 541 - 661	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systemsbp 919-1039)
Bp 662 - 1487	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1488 - 1495	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems) bp 1866-1873)
Bp 1496 – 1524	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1525 – 2733	Transposon, modified from Tn10 GenBank Accession#J01829 Bp 108-1316
Bp 2734 – 3085	Putative PolyA from vector pNK2859
Bp 3086 – 3128	Lambda DNA from pNK2859
Bp 3129 – 3198	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3299 – 3369	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3370 - 3392	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737

Bp 3393 - 5085 Lysozyme Matrix Attachment Region (MAR)from chicken gDNA (GenBank Accession #X98408.

Bp 5086 – 5099 Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI

Bp 5100 - 5946 Chicken Ovalbumin Promoter GenBank accession (#J00895 and M24999)

Bp 5947 - 6545 CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)

Bp 6546 - 6623 CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)

Bp 6624 - 6744 CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039

Bp 6745 - 7570 CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)

Bp 7571 - 7584 CMV Immediate Early Gene, Partial Exon 2 Sal I Restriction Enzymesite from pGWIZ Multiple cloning site (pGWIZ, Gene Therapy Systems)bp1866-1879)

Bp 7585 - 7647 Conalbumin Signal Sequence including Kozak (7585-7590)(GenBank Accession #Y00407)

Bp 7648 - 7704 3xFlag without a Rotational Spacer (Sigma)

Bp 7705 - 7719 Enterokinase Cleavage Site

Bp 7720 - 8295 Codon Optimized human Growth Hormone (GenBank Accession # V00520 bp. 140-715)

Bp 8296 - 9217 Chicken Ovalbumin Regular length Poly A(Genbank accession # J00895 and X01422.)

Bp 9218 - 9264 Cloning Site Extension from pTn X-MCS, Pac I thru Bsi WI.

Bp 9265 - 9577 HSV-TK polyA from pS65TC1 bp 3873-3561

BP 9678 - 10208 Puromycin resistance gene from pMOD PURO (invivoGen)

Bp 10209 - 10600 SV40 promoter from pS65TC1, bp 2232-2617

Bp 10601 - 12305 Lysozyme Matrix Attachment Region (MAR)

Bp 12306 – 12375 70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)

Bp 12376 - 12418 Lambda DNA from pNK2859

Bp 12419 - 14623 pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO: 37 Vector ID# 283 – HPvs1/ CMViA/ CAss/ cohGH(mat/ OPA in pTn-10 PURO-MAR Flanked BV

Bp 1 – 132 Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135

Bp 133 – 148 pGWIZ base vector (Gene Therapy Systems) bp 229-244

Bp 149 - 747 CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)

Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 3016 – 3367	Putative PolyA from vector pNK2859
Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3481 – 3651	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 5367	Chicken Lysozyme Matrix Attachment region (MAR) from gDNA
Bp 5368 – 5381	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp 5382 - 6223	Chicken Ovalbumin promoter from gDNA (Genbank Accession #J00895 bp 421-1261)
BP 6224 - 6827	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)with 5' EcoRI RE site
Bp 6828 - 6905	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-899, CTC, 900-918)
Bp 6906 - 7026	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
BP 7027 - 7852	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 7853 - 7860	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 7861 - 7929	Conalbumin Signal Peptide (Genbank #Y00407 bp 340-385, 1699-1715) with 5'SalI RE site. Kozak sequence (Bp 7867-7872)
Bp 7930 - 8511	Human Growth Hormone-codon optimized (GenBank #V00520 bp 140-715) with 5'AfeI RE site
Bp 8512 - 9433	Chicken Ovalbumin polyA from gDNA (GenBank #J00895 bp 8260-9175) with 5'AatII RE site
Bp 9434 – 9480	MCS extension from pTN-MCS, PacI thru BsiWI

Bp 9481 – 9793	HSV-TK polyA from pS65TC1 bp 3873-3561
BP 9794 - 10424	Puromycin resistance gene from pMOD PURO (invivoGen)
Bp 10425 - 10816	SV40 promoter from pS65TC1, bp 2617-2232 with 5' MluI RE site
Bp 10817 - 12521	Chicken Lysozyme Matrix Attachment region (MAR) from gDNA
Bp 12522 - 12591	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 12592 - 12634	Lambda DNA from pNK2859
Bp 12635 - 14839	pBluescriptII sk(-) base vector (Stratagene, INC)

SEQ ID NO: 38 Vector ID# 268 – HPvs1/ CMViA/ CAss(-3aa)/ cohGH(mat/ OPA in pTn-10 PURO-MAR Flanked BV

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 3016 – 3367	Putative PolyA from vector pNK2859
Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3481 – 3651	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 5367	Chicken Lysozyme Matrix Attachment region (MAR) from gDNA
Bp 5368 – 5381	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp 5382 - 6223	Chicken Ovalbumin promoter from gDNA (Genbank Accession #J00895 bp 421-1261)
BP 6224 - 6827	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)with 5' EcoRI RE site

Bp 6828 - 6905	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-899, CTC, 900-918)
Bp 6906 - 7026	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
BP 7027 - 7852	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 7853 - 7860	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 7861 - 7926	Conalbumin Signal Peptide (Genbank #Y00407 bp 340-385, 1699-1712) with 5'SalI RE site. Kozak sequence (Bp 7867-7872)
Bp 7927 - 8502	Human Growth Hormone-codon optimized (GenBank Accession #V00520 bp 140-715)
Bp 8503 - 9424	Chicken Ovalbumin polyA from gDNA (GenBank #J00895 bp 8260-9175) with 5'AatII RE site
Bp 9425 – 9471	MCS extension from pTN-MCS, PacI thru BsiWI
Bp 9472 – 9784	HSV-TK polyA from pS65TC1 bp 3873-3561
BP 9785 - 10415	Puromycin resistance gene from pMOD PURO (invivoGen)
Bp 10416 - 10807	SV40 promoter from pS65TC1, bp 2617-2232 with 5' MluI RE site
Bp 10808 - 12512	Chicken Lysozyme Matrix Attachment region (MAR) from gDNA
Bp 12513 - 12582	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 12583 - 12629	Lambda DNA from pNK2859
Bp 12630 - 14830	pBluescriptII sk(-) base vector (Stratagene, INC) bp 761-2961

SEQ ID NO:39 Vector ID# 312 – HPvs1/ CMViA/ CAss(-2aa)3Xent/ cohGH(mat/ OPA in pTn-10 PURO-MAR Flanked BV

Bp 1 – 132	Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp 4-135
Bp 133 – 148	pGWIZ base vector (Gene Therapy Systems) bp 229-244
Bp 149 - 747	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)
Bp 748 - 822	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-918)
Bp 823 - 943	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
Bp 944 - 1769	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 1770 - 1777	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)

Bp 1778 – 1806	TN10 DNA, 3'end from Genbank Accession #J01829 bp79 - 107
Bp 1807 – 3015	Transposon, modified from Tn10 GenBank Accession #J01829 Bp 108-1316
Bp 3016 – 3367	Putative PolyA from vector pNK2859
Bp 3368 – 3410	Lambda DNA from pNK2859
Bp 3411 – 3480	70bp of IS10 left from Tn10 (GenBank Accession #J01829 Bp 1-70)
Bp 3481 – 3651	pBluescriptII sk(-) base vector (Stratagene, INC)
Bp 3652 - 3674	Multiple cloning site from pBluescriptII sk(-)thru NotI, Bp 759-737
Bp 3675 - 5367	Chicken Lysozyme Matrix Attachment region (MAR) from gDNA
Bp 5368 – 5381	Multiple Cloning Site Extension from pTn X-MCS, XhoI thru AscI
Bp 5382 - 6222	Chicken Ovalbumin promoter from gDNA (Genbank Accession #J00895 bp 421-1261)
BP 6223 - 6827	CMV Enhancer (vector pGWIZ, Gene Therapy Systems bp 245-843)with 5' EcoRI RE site
Bp 6828 - 6905	CMV Promoter (vector pGWIZ, Gene Therapy Systems bp 844-899, CTC, 900-918)
Bp 6906 - 7026	CMV Immediate Early Gene, Exon 1 (vector pGWIZ, Gene Therapy Systems bp 919-1039)
BP 7027 - 7852	CMV Intron A (vector pGWIZ, Gene Therapy Systems bp 1040-1865)
Bp 7853 - 7860	CMV Immediate Early Gene, Partial Exon 2 (pGWIZ,Gene Therapy Systems)bp 1866-1873)
Bp 7861 - 7929	Conalbumin Signal Peptide (Genbank #Y00407 bp 340-385, 1699-1715) with 5'SalI RE site. Kozak sequence (Bp 7867-7872)
Bp 7930 - 7980	3xFlag
Bp 7981 - 7995	Enterokinase Cleavage Site
Bp 7996 - 8571	Human Growth Hormone-codon optimized (GenBank #V00520 bp 140-715) with 5'AfeI RE site
Bp 8572 - 9493	Chicken Ovalbumin polyA from gDNA (GenBank #J00895 bp 8260-9175) with 5'AatII RE site
Bp 9494 – 9523	MCS extension from pTN-MCS, PacI thru BsiWI
Bp 9524 – 9836	HSV-TK polyA from pS65TC1 bp 3873-3561
BP 9837 - 10467	Puromycin resistance gene from pMOD PURO (invivoGen)
Bp 10468 - 10853	SV40 promoter from pS65TC1, bp 2617-2232 with 5' MluI RE site

Bp 10854 - 12564	Chicken Lysozyme Matrix Attachment region (MAR) from gDNA
Bp 12565 - 12634	70 bp of IS10 from Tn10 (GenBank Accession #J01829 Bp 70-1)
Bp 12635 - 12677	Lambda DNA from pNK2859
Bp 12678 - 14882	pBluescriptII sk(-) base vector (Stratagene, INC)

In one embodiment, the present application provides a novel sequence comprising a promoter, a gene of interest, and a poly A sequence. Each of these novel sequences may be identified from the annotations for each expression vector shown above, and also as sequences within the sequence listing for each expression vector. The specific bases of these novel sequences are provided in Table 3 below for each expression vector SEQ ID NOs: 17 to 39.

Table 3

hGH Vectors		
SEQ ID NO	Begin	End
17 x	4937	10038
18 x	4937	9660
19 x	4937	10044
20 x	4932	12362
21 x	4925	12356
22 sh	4925	11387
23 x	4937	10533
24 x	4931	10527
25 x	4623	10219
26 x	5382	10978
27 sy	4929	7723
28 sy	5374	8168
29 sy	5374	8168
30 x	4937	10533
31 x	5382	10484
32 sh	5377	9499
33 sh	4789	8906
34 sh	5100	9217
35 sh	4789	8906
36 sh	5100	9217
37 sh	5382	9433
38 sh	5382	9424
39 sh	5382	9493

X indicates extended ovalbumin polyA.

sh indicates short ovalbumin polyA.

sy indicates synthetic ovalbumin polyA.

E. Methods of In Vivo Administration

The polynucleotide cassettes may be delivered through the vascular system to be distributed to the cells supplied by that vessel. For example, the compositions may be administered through the cardiovascular system to reach target tissues and cells receiving blood supply. In one embodiment, the compositions may be administered through any chamber of the heart, including the right ventricle, the left ventricle, the right atrium or the left atrium. Administration into the right side of the heart may target the pulmonary circulation and tissues supplied by the pulmonary artery. Administration into the left side of the heart may target the systemic circulation through the aorta and any of its branches, including but not limited to the coronary vessels, the ovarian or testicular arteries, the renal arteries, the arteries supplying the gastrointestinal and pelvic tissues, including the celiac, cranial mesenteric and caudal mesenteric vessels and their branches, the common iliac arteries and their branches to the pelvic organs, the gastrointestinal system and the lower extremity, the carotid, brachiocephalic and subclavian arteries. It is to be understood that the specific names of blood vessels change with the species under consideration and are known to one of ordinary skill in the art. Administration into the left ventricle or ascending or descending aorta supplies any of the tissues receiving blood supply from the aorta and its branches, including but not limited to the testes, ovary, oviduct, and liver. Germline cells and other cells may be transfected in this manner. For example, the compositions may be placed in the left ventricle, the aorta or directly into an artery supplying the ovary or supplying the fallopian tube to transfect cells in those tissues. In this manner, follicles could be transfected to create a germline transgenic animal. Alternatively, supplying the compositions through the artery leading to the oviduct would preferably transfect the tubular gland and epithelial cells. Such transfected cells could manufacture a desired protein or peptide for deposition in the egg white. Administration of the compositions through the left cardiac ventricle, the portal vein or hepatic artery would target uptake and transformation of hepatic cells. Administration may occur through any means, for example by injection into the left ventricle, or by administration through a cannula or needle introduced into the left atrium, left ventricle, aorta or a branch thereof.

Intravascular administration further includes administration in to any vein, including but not limited to veins in the systemic circulation and veins in the hepatic portal circulation. Intravascular administration further includes administration into the cerebrovascular system, including the carotid arteries, the vertebral arteries and branches thereof.

Intravascular administration may be coupled with methods known to influence the permeability of vascular barriers such as the blood brain barrier and the blood testes barrier, in

order to enhance transfection of cells that are difficult to affect through vascular administration. Such methods are known to one of ordinary skill in the art and include use of hyperosmotic agents, mannitol, hypothermia, nitric oxide, alkylglycerols, lipopolysaccharides (Haluska et al., Clin. J. Oncol. Nursing 8(3): 263-267, 2004; Brown et al., Brain Res., 1014: 221-227, 2004; Ikeda et al., Acta Neurochir. Suppl. 86:559-563, 2004; Weyerbrock et al., J. Neurosurg. 99(4):728-737, 2003; Erdlenbruch et al., Br. J. Pharmacol. 139(4):685-694, 2003; Gaillard et al., Microvasc. Res. 65(1):24-31, 2003; Lee et al., Biol. Reprod. 70(2):267-276, 2004)).

Intravascular administration may also be coupled with methods known to influence vascular diameter, such as use of beta blockers, nitric oxide generators, prostaglandins and other reagents that increase vascular diameter and blood flow.

Administration through the urethra and into the bladder would target the transitional epithelium of the bladder. Administration through the vagina and cervix would target the lining of the uterus and the epithelial cells of the fallopian tube.

The polynucleotide cassettes may be administered in a single administration, multiple administrations, continuously, or intermittently. The polynucleotide cassettes may be administered by injection, via a catheter, an osmotic mini-pump or any other method. In some embodiments, a polynucleotide cassette is administered to an animal in multiple administrations, each administration containing the polynucleotide cassette and a different transfecting reagent.

In a preferred embodiment, the animal is an egg-laying animal, and more preferably, an avian, and the transposon-based vectors comprising the polynucleotide cassettes are administered into the vascular system, preferably into the heart. The vector may be injected into the venous system in locations such as the jugular vein and the metatarsal vein. In one embodiment, between approximately 1 and 1000 μg, 1 and 200 μg, 5 and 200 μg, or 5 and 150 μg of a transposon-based vector containing the polynucleotide cassette is administered to the vascular system, preferably into the heart. In a chicken, it is preferred that between approximately 1 and 300 μg, or 5 and 200 μg are administered to the vascular system, preferably into the heart, more preferably into the left ventricle. The total injection volume for administration into the left ventricle of a chicken may range from about 10 μl to about 5.0 ml, or from about 100 μl to about 1.5 ml, or from about 200 μl to about 1.0 ml, or from about 200 μl to about 800 μl. It is to be understood that the total injection volume may vary depending on the duration of the injection. Longer injection durations may accommodate higher total volumes. In a quail, it is preferred that between approximately 1 and 200 μg, or between approximately 5 and 200 μg are administered to the vascular system, preferably into the heart, more preferably into the left ventricle. The total injection volume for administration into the left ventricle of a quail may range from about 10 μl to about 1.0 ml, or

from about 100 µl to about 800 µl, or from about 200 µl to about 600 µl. It is to be understood that the total injection volume may vary depending on the duration of the injection. Longer injection durations may accommodate higher total volumes. The microgram quantities represent the total amount of the vector with the transfection reagent.

In another embodiment, the animal is an egg-laying animal, and more preferably, an avian. In one embodiment, between approximately 1 and 150 µg, 1 and 100 µg, 1 and 50 µg, preferably between 1 and 20 µg, and more preferably between 5 and 10 µg of a transposon-based vector containing the polynucleotide cassette is administered to the oviduct of a bird. In a chicken, it is preferred that between approximately 1 and 100 µg, or 5 and 50 µg are administered. In a quail, it is preferred that between approximately 5 and 10 µg are administered. Optimal ranges depending upon the type of bird and the bird's stage of sexual maturity. Intraoviduct administration of the transposon-based vectors of the present invention result in a PCR positive signal in the oviduct tissue, whereas intravascular administration results in a PCR positive signal in the liver, ovary and other tissues. In other embodiments, the polynucleotide cassettes is administered to the cardiovascular system, for example the left cardiac ventricle, or directly into an artery that supplies the oviduct or the liver. These methods of administration may also be combined with any methods for facilitating transfection, including without limitation, electroporation, gene guns, injection of naked DNA, and use of dimethyl sulfoxide (DMSO). U.S. Patent No. 7,527,966, U.S. Publication No. 2008/0235815, and PCT Publication No.WO 2005/062881 are hereby incorporated by reference in their entirety.

The following examples will serve to further illustrate the present invention without, at the same time, however, constituting any limitation thereof. On the contrary, it is to be clearly understood that resort may be had to various embodiments, modifications and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the invention.

EXAMPLE 1

Experiments to Compare Vectors Containing Hybrid Promoters

These experiments were conducted to determine if the vectors were functioning. No attempt was made to select or clone these cells for long term expression. In the first two experiments, the first objective of each experiment was to determine whether the new promoters would function in tubular gland and LMH2A cells, respectively. A second objective of the second experiment was to determine if the promoters were estrogen responsive. Estrogen responsiveness could not be

tested in tubular gland cells (first experiment) because estrogen cannot be removed from these cells long enough for them to return to a basal level. If this is done in culture, the cells lose their ability to respond to estrogen. The results from this experiment showed that all of the hybrid promoters (SEQ ID NOs:14 and 15, and base pairs 4937 to 6465 of SEQ ID NO:19) inserted into backbone vectors SEQ ID NOs:17, 18, and 19, respectively, functioned in tubular gland cells with versions SEQ ID NOs:17 and 18 expressing the most protein *in vitro*. In the second experiment, LMH2A cells were transfected with each of the hybrid promoter vectors SEQ ID NOs:17, 18, and 19. Duplicate flasks were set up for each vector so expression in the absence and presence of estrogen could be determined. The most pronounced effect in estrogen responsiveness was seen in the M3 (3^{rd} media sample) taken 6 days after transfection.
The results are summarized below:

1) Each of the hybrid promoters (SEQ ID NOs:14 and 15, and base pairs 4937 to 6465 of SEQ ID NO:19) inserted into backbone vectors SEQ ID NOs:17, 18, and 19, respectively, worked in tubular gland cells and LMH2A cells;

2) SEQ ID NOs:17 and 18 containing hybrid promoters SEQ ID NOs:14 and 15 (versions 1 and 2, respectively) expressed best in both cell types;

3) In LMH2A cells, all 3 promoters appeared to be estrogen responsive; and,

4) Addition of the ovalbumin estrogen responsive elements did not confer tissue specificity *in vitro*.

The third experiment was conducted in LMH2A cells in order to compare the hybrid promoters to the original CMV promoter and estimate the amount of protein being expressed. Again, no attempt was made to select or clone these cells for long term expression, only transient expression was examined. The results differed from our normal expression pattern. Normally, the M2 and M3 media samples gave the highest rate of expression, but in this experiment, M1 was highest. This was not due to the hybrid promoters since the original CMV promoter was included in this experiment and produced the same expression pattern. Regardless, the hybrid promoter version 1 and 2 (SEQ ID NOs:14 and 15 in backbone vectors SEQ ID NOs:17 and 18, respectively), expressed protein at a rate nearly double that of the original CMV promoter in M1 media and three times that of the CMV promoter in M2 media. The original CMV promoter outperformed hybrid promoter version 3 almost 3:1 in M1 media and about 5:1 in M2 media.

EXAMPLE 2

Transfection of LMH2A and Chicken Tubular Gland Cells with CMV/Oval$_p$-3xF-hGH

Experiments were conducted to test three vectors, SEQ ID NOs:17, 18, and 19, containing different versions of the hybrid CMV/Oval promoter (SEQ ID NOs:14, 15, and bp 4937-6465 of SEQ ID NO:19, respectively) with the 3xFlag hGH cassette (OVep/CMV intron A/Con SS/3xFlag/hGH/OV ext PA). Chicken tubular gland (ChTG) cells were tested in the first experiment, and LMH2A cells were used for the second experiment. Our standard protocol for each cell type was followed. The transfection scheme is shown below.

Table 4

1121 Flask ChTG	1122 Flask LMH2A	Vector	SEQ ID NO:	Promoter	SEQ ID NO:
C1	C1 & C2	N/A	--	N/A	--
T1	T1 & T2	153	17	Vs. 1	14
T2	T3 & T4	173	18	Vs. 2	17
T3	T5 & T6	174	19	Vs. 3	bp 4937-6465 of SEQ ID NO:19

The tubular gland cells were fed with media containing 1x estrogen, insulin and corticosterone prior to transfection (standard protocol). Stock solutions of these supplements are made at 1000X. 1X in media gives the final concentrations: Beta-estradiol: 200 μM; corticosterone: 10 μM; and, insulin: 50 ng/ml. These are the concentrations used for tubular gland cells. When LMH2A cells are stimulated with estrogen, 50x B-estradiol was used and 1x insulin, but no corticosterone. Media was collected 3 days (M1) and 6 days (M2) post transfection. Cells were harvested on the sixth day.

In the LMH2A cells, standard Waymouth's + 10% fetal calf serum (FCS) was used for transfection. After 24 hours, media was collected (M1), and C1, T1, T3 and T5 were fed with Waymouth's + 5% FCS. C2, T2, T4 and T6 were fed the same plus 50x estrogen/1x insulin. This was done to determine if the steroids affected promoter activity. Media was collected again at 3 (M2) and 6 (M3) days post transfection. Cells were harvested on day 6.

Figure 3 shows the GH ELISA results for the chicken tubular gland cells with the three versions of the hybrid promoter. Clearly, Version 1 (T1 (SEQ ID NO: 17)) of the promoter gives consistently higher readings in these cells under these conditions, followed by Version 2 (T2 (SEQ ID NO:18)), with Version 3 (T3 (SEQ ID NO:19) giving the lowest readings. The L1**

samples were run on a different plate than the rest of the samples, and a positive control run on both plates gave slightly lower readings on the plate with the lysate samples. Accordingly, it was not possible to compare absolute values between the lysates and the media samples. Also note that 90-minute readings were used for these comparisons, because later readings 'maxed out' the reader. No attempt was made to quantitate the protein content of these samples.

Figure 4 shows the 90-minute ELISA readings from Expt. I122, done in LMH2A cells. Note that these readings were done on 1:500 dilutions of the original samples. T1, T3, and T5 are Versions 1, 2, and 3 (SEQ ID NOs:I4, I5, and base pairs 4937-6465 of SEQ ID NO:I9) in the vectors shown as SEQ ID NOs:I7, I8, and 19, respectively, without estrogen. T2, T4, and T6 are the same, with estrogen. Therefore, when looking at the graphs in Figure 4, each pair of bars represents a single promoter version, with the first bar being that version without estrogen, and the second bar representing the sample run with estrogen. For each of MI, M2, M3, and LI in the next two figures, the T1 through T6 results are shown in order from left to right. As in the tubular gland cells, SEQ ID NO:17 gives the highest readings, followed by SEQ ID NO:I8, with SEQ ID NO:I9 giving the lowest readings. This experiment, however, was also designed to examine the estrogen-responsiveness of these hybrid promoters in LMH2A cells. It does appear that all 3 promoters were responsive to estrogen in these cells, with the most statistically significant differences at the M3 time point in which all samples were statistically different from each other except for T3 and T6 ($p<0.05$).

Figure 5 shows the estimated protein concentrations, calculated from the 90-minute readings, for each sample shown above. There were essentially no differences between this graph and the one above. Clearly, in both cell types, the vector containing Version I (SEQ ID NO:I7) produced the most GH, followed by the vector containing Version 2 (SEQ lD NO:18) and then by the vector containing Version 3 (SEQ ID NO:I9). All 3 appeared to be estrogen responsive, at least in LMH2A cells, and the differences became more pronounced with time. All 3 of these constructs use the CMV core promoter.

The hybrid promoters were prepared independently and then cloned upstream of the CMV intron A. (SEQ ID NO:I4) Version 1, the highest producer, had the CMV enhancer directly preceding the promoter, in its 'normal' position, and this combination appeared to give the highest production. A portion of the OVAL 5' upstream region containing sequences identified as the steroid dependent response element (SDRE) and negative response element (NRE) preceded the CMVenh promoter, and seemed to confer steroid responsiveness on the CMVpr in LMH2A cells. SEQ ID NO:I5 Version 2 was almost the same structure, except that the NRE was moved to a position between the CMV enhancer and promoter. This significantly reduced total production. (Base pairs 4937-6465 of SEQ ID NO:I9) Version 3 placed the entire

Ch OVAL SDRE through NRE sequence between the CMV enhancer and promoter, further reducing overall protein production by the promoter.

EXAMPLE 3

Hybrid-hGH Vectors in LMH2A Cells

This experiment was conducted to test three vectors (SEQ ID NOs:17, 18, and 19) with different versions of the CMV/Oval hybrid promoter. All vectors were based on the hGH gene from vector 171 (SEQ ID NO:27). LMH2A cells were seeded to 5 T25s flasks (25 cm^2) the day prior to transfection. One flask was used for control, and the other four were transfected using 2 μg DNA and Fugene 6 (DNA:Fugene=1:6) following our standard protocol:

T1: 171 (SEQ ID NO:27)

T2: 153 (hybrid promoter v1) (SEQ ID NO:17)

T3: 173 (hybrid promoter v2) (SEQ ID NO:18)

T3: 174 (hybrid promoter v3) (SEQ ID NO:19)

Media was collected and the cells were fed every 2-3 days.

Figure 6 illustrates the protein quantification (ng/well) from the ELISA results. The M1 and M2 samples were diluted 1:5000, and the M3 and M4 samples were diluted 1:500 for the ELISA assay.

The M1 and M2 samples were also tested at the same 1:5000 dilutions and some varied by as much as 35%. The comparison of these readings is shown in Table 5 below. In addition, many of the M3 and M4 samples were below the standard curve. Based on an earlier result, both vectors SEQ ID NO:17 and 18 containing versions 1 and 2 of the hybrid promoter were better than SEQ ID NO:27 in the first two media samples.

Table 5

1st ELISA	ng/ml	2nd ELISA	ng/ml	% Difference
T1.M1 1:5000	4961.9	T1.M1 1:5000	5030.7	1.39%
T2.M1 1:5000	9212.1	T2.M1 1:5000	9102.9	-1.18%
T3.M1 1:5000	9789.6	T3.M1 1:5000	11150.4	13.90%
T4.M1 1:5000	1497.0	T4.M1 1:5000	1788.9	19.50%
T1.M2 1:5000	811.2	T1.M2 1:5000	1095.9	35.09%
T2.M2 1:5000	3049.9	T2.M2 1:5000	3005.2	-1.47%
T3.M2 1:5000	2685.6	T3.M2 1:5000	2867.8	6.79%
T4.M2 1:5000	0.0	T4.M2 1:5000	274.6	?

EXAMPLE 4

New hGH Vectors

This experiment was designed to test several new vectors in cell culture. These are variations on Series A containing the ovalbumin promoter and intron or Series B containing the ovalbumin promoter and the CMV intron A.

Series A Vectors (Figure 7) included the following:

T1: The original (Ovenh prom/Ov Ver 5(OVg + O ia)/nrs 3XF/co hGH/OV XLpA) in the HS4 flanked backbone vector. (# 157) (SEQ ID NO:20)

T2: Same cassette, but in the pTn10HFB backbone vector. (# 181) (SEQ ID NO:21)

T3: Same as T2, but with the original, shorter OVpA. (# 170) (SEQ ID NO:22)

Note that although there was little difference between these vectors in the early samples, by the M3, and especially the M4 samples, the T3 (shorter polyA) vector (SEQ ID NO:22) gave significantly higher protein readings, suggesting that it was maintaining higher levels of protein production longer than the others (Figure 7).

Series B Vectors (Figure 8) included the following:

T4: The original (OVep/CMV IA/CONss/(-rs)3xFlag/hGH/OV XLpA) in the HS4-flanked backbone vector (# 159) (SEQ ID NO:23)

T5: Same cassette, but in the pTn10 HS4-flanked backbone vector (# 202) (SEQ ID NO:24)

T6: Same cassette, but in the LysRep Ver 2 backbone vector (# 203) (SEQ ID NO:25)

T7: Same cassette, but in the MAR-flanked backbone vector (# 204) (SEQ ID NO:26)

T8: The original in the HS4-flanked BV (VID# 171) (SEQ ID NO:27).

As in the previous series, all are quite similar in the early samples, but note that in M3, the LysRep and MAR vectors (T6 and T7 (SEQ ID NOs:25 and 26, respectively)) actually continued to increase GH production, and maintain higher levels than the other vectors for the duration of the experiment (Figure 8). Figure 9 includes all samples, to provide a comparison of the difference in total protein production between the Series A and Series B vectors.

EXAMPLE 5

Test Transfection of hGH Vectors

This experiment compared two versions of new hGH vectors. Both of these vectors were in the TnPuroMAR backbone. However, one of these vectors, (T1 #217) (SEQ ID NO:28), had the hGH expression cassette inserted tail-to-tail (polyA-to-polyA) with the puromycin expression cassette, while the other, (T2 #240) (SEQ ID NO:29), had the hGH cassette inserted head-to-tail with the puromycin cassette, such that the CMV promoter of the hGH cassette immediately followed the polyA of the puromycin cassette, with both cassettes reading in the same direction.

Although these vectors were in a puromycin-selectable backbone, they were not exposed to any selection pressure in this experiment. Figure 10 shows only the first two media samples. Clearly, the tail-to-tail vector (T1, SEQ ID NO:28) expressed much higher levels of 3xFlag-GH in this experiment. (The actual values reported by protein are shown in the table below.)

Table 6

	3xFlag-GH (mg/ml)
T1 (SEQ ID NO:25).M1 (48 hours) 1:1000	6.03
T1 (SEQ ID NO:25)..M2 (96 hours) 1:1000	0.35
T2 (SEQ ID NO:26)..M1 (48 hours) 1:10K	52.48
T2 (SEQ ID NO:26)..M2 (96 hours) 1:10K	15.47

EXAMPLE 6

GH Protein Expression Using Transposon Based Expression Vectors.

The disclosed expression vectors have been assayed for their ability to produce GH as discussed above, and in several different cell cultures. Typical results for the expression vectors are shown in Table 7.

Table 7

Vector number	**Cell type**	**Amount of protein**
153	TG	15 μg/ml
153	LMH2A	10 μg/ml
157	TG	5.8 μg/ml
159	TG	13.1 μg/ml
170	TG	3.6 μg/ml
171	TG	4.35 abs @ 120 min
171	LMH2A	40.1 μg/ml
171	hMyeloma	.35 abs @ 180 min
173	TG	5.6 μg/ml
173	LMH2A	27 μg/ml
174	TG	3.1 μg/ml
174	LMH2A	12.8 μg/ml
181	TG	3.1 μg/ml
202	TG	13.7 μg/ml
203	TG	14 μg/ml
204	TG	14 μg/ml
235	LMH2A	45.3 μg/ml
235	LMH	31.8 μg/ml
235	APRE	3.6 μg/ml
235	CHO-K1	3.3 μg/ml
268	LMH	8.7 μg/ml
268	LMH2A	8.5 μg/ml
283	LMH	5.1
283	LMH2A	5.0 μg/ml
230	LMH2A	38.26 μg/ml
230	LMH	27.89 μg/ml

EXAMPLE 7

TnMAR

There are two basic inventions that are presented together because of how they work in concert with each other to increase gene expression. The first was developed in an effort to overcome gene silencing in chickens. We have developed several vectors that had insulator elements "in-board" of the insertion sequences of the transposon vector. These include HS4 elements, Lys Rep elements (Lysozyme Replicator), Lys Rep/HS4 elements in combination, and MAR (Matrix Attachment Region) elements (schematically represented in Figure 11 and shown in different vectors described herein, see Appendix). The goal in designing vectors containing these elements was to identify a system that would prevent our transgene from being bound and silenced by chromatin. While not wanting to be bound by the following statement, in theory, these elements are supposed to inhibit or at least minimize gene silencing. Each of these has been tested in cell culture. To date, the vector containing the MAR and LysRep vectors worked the best with MAR slightly better than LysRep.

A second embodiment of this invention are two hybrid promoters that consist of elements from the constitutive CMV promoter and the estrogen inducible ovalbumin promoter. The goal of designing these promoters was to couple the high rate of expression associated with the CMV promoter with the estrogen inducible function of the ovalbumin promoter. To accomplish this goal, three hybrid promoters, designated versions 1-3 (SEQ ID NOs:14, 15, and base pairs 4937-6465 of SEQ ID NO:19, respectively) (Figures 1-2, and Appendix), were designed, built, placed into backbone vectors and tested in cell culture. Of the three, only versions 1 and 2 provided high rates of expression; version 3 provided less and was not considered for further work in cell culture.

Unexpectedly, in cell culture the TnMAR vector provided significantly more GH than previous vectors we had developed, with the exception of the LysRep vectors. Likewise, the hybrid promoters version 1 (SEQ ID NO:14) and 2 (SEQ ID NO:15) out performed the original CMV promoter by a range of 25 to 50% with most samples approaching 50%.

EXAMPLE 8

Immunocytochemical Detection of Human Growth Hormone in transfected LMH2A cells

Transfected LMH2A cells were seeded to one well of gelatin-coated 2-well chamber slides and grown in the presence of puromycin. LMH2A control cells (non-transfected) were grown in the second well of each chamber slide without antibiotic. Cells were fixed with 4% neutral buffered formalin and stained using the following method. Cells were permeabilized in 2.5% cold methanol for 5 min. Blocking buffer included 2.5% normal horse serum in phosphate

buffered saline and 0.01% Triton X-100 for 20 min. Primary antiserum employed were anti-3X flag-fluorescein isothiocyanate (FITC) 8ug/ml or mouse anti-hGH ab9821 1:1000 dilution (1ug/ml) for 60 min. Secondary antisera were anti-mouse Ig-FITC 1:500 or anti-mouse Ig-rhodamine 1:500 for 30 min.

Fluorescent labeling of cells expressing hGH was observed with a Zeiss Axiovert 200 Microscope. Non-transfected LMH2A control cells showed some autofluorescence but no staining with FITC-conjugated antibody, by either direct detection with anti-3xFlag-FITC or indirect detection using mouse anti-hGH with anti-mouse Ig-FITC or rhodamine secondary antibody. The puromycin-selected transfected LMH2A cells showed a majority of the cell population specifically labeled with the direct and indirect methods of detection listed above, demonstrating hGH synthesis in the transfected LMH2A cells.

EXAMPLE 9

Perfusion of LMH2A Cells in AutoVaxID

The AutoVaxID cultureware (Biovest) was installed and the Fill-Flush procedure was performed following the procedures in the AutoVaxID Operations Manual. The following day, the pre-inoculation procedure and the pH calibration were done. The cultureware was seeded with 10^9 LMH2A cells containing vector #230 (SEQ ID NO:31). The cells had been propagated in Lonza UltraCULTURE media supplemented with cholesterol (Sigma, 50 ug/ml) in 20 gelatin-coated T150 cell culture flasks, and had been dissociated with Accutase (Sigma). They were counted, gently pelleted (600xG for 6 minutes) and resuspended in 50 mls of growth media (Lonza UltraCULTURE containing GlutaMax (Invitrogen) and SyntheChol (1:500), Soy Hydrolysate (1:50), and Fatty Acid Supplement (1:500) (all from Sigma). This is the same media which was included in the "Factor" bags for the AutoVaxID, used for the EC (extra-capillary) media. A 10 L bag of Lonza UltraCULTURE media (with GlutaMax) was used initially for the IC (intra-capillary) media. This was designed to give the cells a richer media for the first 7-10 days, to allow them to become established quickly in the hollow fiber system. After this bag was exhausted, the IC media was switched to DMEM/F12 (also including GlutaMax), also purchased from Lonza. This media was purchased in 50L drums, and was removed from the cold room and allowed to warm to room temperature before being connected to the system. The AutoVaxID system was placed under Lactate Control, and pump rates were modified and daily tasks performed, as specified by the AutoVaxID Operating Procedures Manual, provided by the manufacturer (Biovest).

Six days later cells could clearly be seen growing on the hollow fibers in the bioreactor. Up until this time, there was ample evidence that the cells were growing and metabolizing in the

system; the Lactate Controller had been increasing the media pump rate regularly in order to keep the lactate levels below the setpoint, and the pH Controller had been continually decreasing the percentage of CO_2 in the gas mix, indicating that the cells were producing increasing amounts of acidic metabolic products. After the IC media was changed from the Lonza UltraCULTURE media to the DMEM/F12, however, the metabolic rate of the cells appeared to slow dramatically, to the point where the Lactate Controller had slowed the media pumps all the way to baseline levels, and the lactate levels were still dropping. Samples were taken for protein analysis 4 days later (Day 11). Samples were taken from the EC media (showing current production) from the Harvest Bag (showing accumulated production) and from the IC media (showing any protein which crossed the membrane and was lost in the wasted media). By four days later (Day 15), there was both visual and metabolic evidence that the cells were growing, so cycling was initiated. For the next week, regular sampling was continued, and cells appeared to be growing and metabolizing normally, although it was becoming physically difficult to pull samples from the EC sample port. The run was allowed to continue until Day 32, although cycling times became greatly extended. Final samples were taken, and the run was ended. All samples were analyzed for proteins. Figure 12 shows the amounts of 3xFlag-hGH in each of the EC and harvest samples (Day 11, 15, 18, 20, 22, and 32). The cells were clearly capable of producing significant amounts of protein in this system.

EXAMPLE 10

Construction of Vectors #133/#159 (SEQ ID NO:30)

The pTopo containing the human growth hormone (hGH) cassette driven by the hybrid promoter version 1 (SEQ ID NO:14) was digested with restriction enzymes Asc I and Pac I (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Digested DNA was purified from restriction enzymes using a Zymo DNA Clean and Concentrator kit (Zymo Research). To insert the growth hormone cassette into the MCS of the p5006 vector (SEQ ID NO:3), the purified hGH DNA and the p5006 vector (SEQ ID NO:3) were digested with Asc I and Pac I, purified as described above, and ligated using a Quick T4 DNA Ligase Kit (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Ligated product was transformed into *E. coli* Top10 competent cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to Invitrogen's protocol. Transformed bacteria were incubated in 0.25ml of SOC (GIBCO BRL, CAT# 15544-042) medium for 1 hour at 37°C before being spread to LB (Luria-Bertani media (broth or agar)) plates supplemented with 100 μg/ml ampicillin (LB/amp plates). These plates were incubated overnight at 37°C, and resulting colonies picked to LB/amp broth for overnight growth at 37°C. Plasmid DNA was isolated using

a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on a U.V. transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size were cultured in at least 250 ml of LB/amp broth and plasmid DNA harvested using a Qiagen Maxi-Prep Kit (column purification) according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). Column purified DNA was used as template for sequencing to verify that the changes made in the vector were the desired changes and that no further changes or mutations occurred. All sequencing was done on a Beckman Coulter CEQ 8000 Genetic Analysis System.

Once a clone was identified that contained the hGH gene, the DNA was isolated by standard procedures. Briefly, *E. coli* containing the plasmid was grown in 500 mL aliquots of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight with shaking. Plasmid DNA was recovered from the bacteria using a Qiagen EndoFree Plasmid Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 µL of Endotoxin free water and stored at -20°C until needed.

EXAMPLE 11

Production of transgenic chicken and quail that successfully passed the hGH transgene through two generations using SEQ ID NO:30

Separate *in vivo* experiments in chicken and quail have demonstrated successful passage of the transgene encoding for hGH contained in SEQ ID NO:30 through two generations. Briefly, germ line cells of both chicken and quail were made transgenic following administration of SEQ ID NO:30 containing a gene encoding for human growth hormone (hGH), into the left cardiac ventricle, the source of the aorta which provides an artery leading to the ovary; 2) These birds were mated with naïve males and the resulting eggs hatched. These chicks (G1 birds) contained the transgene encoding hGH as their blood cells were positive for the transgene encoding hGH or the transgene encoding hGH, and 3) These transgenic progeny (G1 birds) were subsequently bred and their progeny (G2 birds) were positive for the transgene encoding hGH.

Transgenic G1 and G2 quail were generated by injecting females in the left cardiac ventricle. The experiment employed 5, seven week old quail hens. The hens were each injected into the left ventricle, allowed to recover, and then mated with naïve males. Isofluorane was used to lightly anesthetize the birds during the injection procedure. Eggs were collected daily for six days and set to hatch on the 7th day. At about 2 weeks of age, the chicks were bled and DNA harvested as described in a kit protocol from Qiagen for isolating genomic DNA from blood and tissue. PCR was conducted using primers specific to the gene of interest. In both experiments, transgene-positive G1 animals were obtained. These transgene-positive G1 animals were raised

to sexual maturity and bred. The G2 animals were screened at 2 weeks of age and transgenic animals were identified in each experiment. A transposon-based vector (SEQ ID NO:30) containing a gene for a hGH was injected. A total of 85 μg complexed with branched polyethylimine (BPEI) in a 300 μL total volume was used. G1 and G2 quail were positive for the hGH transgene following analysis of blood samples.

Transgenic G1 and G2 chickens were generated by injecting females in the left cardiac ventricle. The experiment was conducted in 20 week old chickens. In the second experiment, the same transposon-based vector (SEQ ID NO:30) containing a gene for a hGH as described above for quail was injected. DNA (complexed to BPEI) was delivered to the birds at a rate of 1 mg/kg body (up to 3 ml total volume) weight by injection into the left cardiac ventricle. Isofluorane was used to lightly anesthetize the birds during the injection procedure. Once the birds recovered from the anesthesia, they were place in pens with mature, naïve males. All eggs were collected for 5 days and then incubated. In the first experiment, the eggs were incubated for about 12 days, candled to check for viable embryos; any egg showing a viable embryo was cracked open and tissue samples (liver) taken from the embryo for PCR. The eggs were allowed to hatch, and a blood sample was taken at two days to test the animals for the presence of the transgene using PCR. Approximately 14% of the chicks were positive for the hGH transgene.

EXAMPLE 12

Production of human growth hormone from transfected tubular gland cells in vitro using SEQ ID NO:30

Oviducts were harvested from 19-22 week old white leghorn hens and cleaned by removing the infundibulum, shell gland, membranes, and blood vessels to leave the magnum portion of the oviduct. The tissue was minced into about 2 mm^2 pieces and dissociated into single or multi-cell clumps using Liberase 2 Blendzyme (Roche). The tubular gland cell population was enriched by centrifuging the dissociated cells on a Percoll SIP gradient and removing the top layer. These cells were allowed to attach in T25 flasks for 24-48 hours in DMEM/F12 Advanced media supplemented with 10% fetal calf serum. Once attached, the media was gently poured off and 2 μg of DNA (containing vector SEQ ID NO:30) was complexed with Fugene-6 in a final volume of 1 ml media was added to the cells and incubated at 37°C for 1-2 hours. After incubation, the DNA/Fugene complex was removed and fresh media added to the flask. Media was harvested every 48 hours (for a total of three harvests designated M1, M2, and M3) and stored at 4°C until the experiment was completed. Once all samples were obtained, an ELISA was conducted using anti-3xFlag antibody. Numerous experiments were conducted. As an example, vector 159 yielded an absorbance of 0.745 compared to the negative control

absorbance of 0.065 and the positive control absorbance of 0.644 (180 min reading at 405 nm). This was the highest absorbance obtained in this experiment. These samples were also used on a Western blot using anti-3xFlag as the detection antibody and a 27 KD band corresponding to the 3xFlag growth hormone was observed to be the same size as the positive control band. These results demonstrate that Vector 133/159 (SEQ ID NO:30) was successfully used to transfect tubular gland cells that synthesized and secreted 3xFlag human growth hormone.

All patents, publications and abstracts cited above are incorporated herein by reference in their entirety. It should be understood that the foregoing relates only to preferred embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the present invention as defined in the following claims.

CLAIMS

1. A vector comprising:

a modified transposase gene operably linked to a first promoter, wherein the nucleotide sequence 3' to the first promoter comprises a modified Kozak sequence, and wherein a plurality of the first twenty codons of the transposase gene are modified from the wild-type sequence by changing the nucleotide at the third base position of the codon to an adenine or thymine without modifying the amino acid encoded by the codon;

a multiple cloning site;

transposon insertion sequences recognized by a transposase encoded by the modified transposase gene, wherein the transposon insertion sequences flank the multiple cloning site; and,

one or more insulator elements located between the transposon insertion sequences and the multiple cloning site.

2. The vector of claim 1 comprising any one of SEQ ID NOs: 2 to 13.

3. The vector of claim 1, wherein the vector comprises any one of SEQ ID NOs: 10 to 13.

4. The vector of claim 1, further comprising a second promoter, wherein the second promoter is SEQ ID NO: 14 or SEQ ID NO: 15.

5. The vector of claim 4, further comprising a gene encoding for growth hormone inserted into the multiple cloning site.

6. The vector of claim 5, wherein the vector comprises any one of SEQ ID NOs: 17 to 39.

7. A promoter comprising chicken ovalbumin promoter regulatory elements in combination with a cytomegalovirus enhancer and a cytomegalovirus promoter.

8. The promoter of claim 7 comprising SEQ ID NO: 14.

9. A promoter comprising a steroid dependent response element, a cytomegalovirus enhancer, a chicken ovalbumin negative response element and a cytomegalovirus promoter.

10. The promoter of claim 9 comprising SEQ ID NO: 15.

11. A transposon-based vector comprising:

a modified transposase gene operably linked to a first promoter, wherein the nucleotide sequence 3' to the first promoter comprises a modified Kozak sequence, and wherein a plurality of the first twenty codons of the transposase gene are modified from the wild-type sequence by

changing the nucleotide at the third base position of the codon to an adenine or thymine without modifying the amino acid encoded by the codon;

one or more genes of interest encoding growth hormone operably-linked to one or more additional promoters, wherein the one or more genes of interest encoding growth hormone and their operably-linked promoters are flanked by transposon insertion sequences recognized by a transposase encoded by the modified transposase gene; and,

one or more insulator elements located between the transposon insertion sequences and the one or more genes of interest encoding growth hormone.

12. The vector of claim 11, wherein the vector comprises any one of SEQ ID NOs: 17 to 39.

13. A method of producing growth hormone comprising:

transfecting a cell with a vector comprising a modified gene encoding for a transposase, a promoter and a gene encoding for growth hormone;

culturing the transfected cell in culture medium;

permitting the cell to release growth hormone into the culture medium;

collecting the culture medium; and,

isolating the growth hormone.

14. The method of claim 13 wherein the vector comprises any one of SEQ ID NOs: 17 to 39.

Figure 1

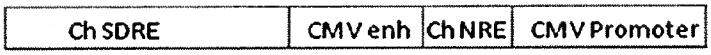


Figure 2

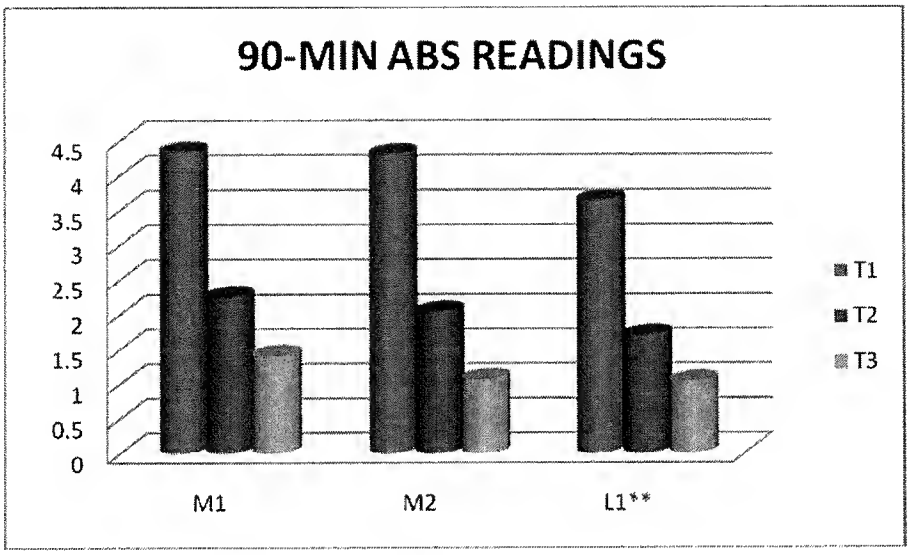


Figure 3

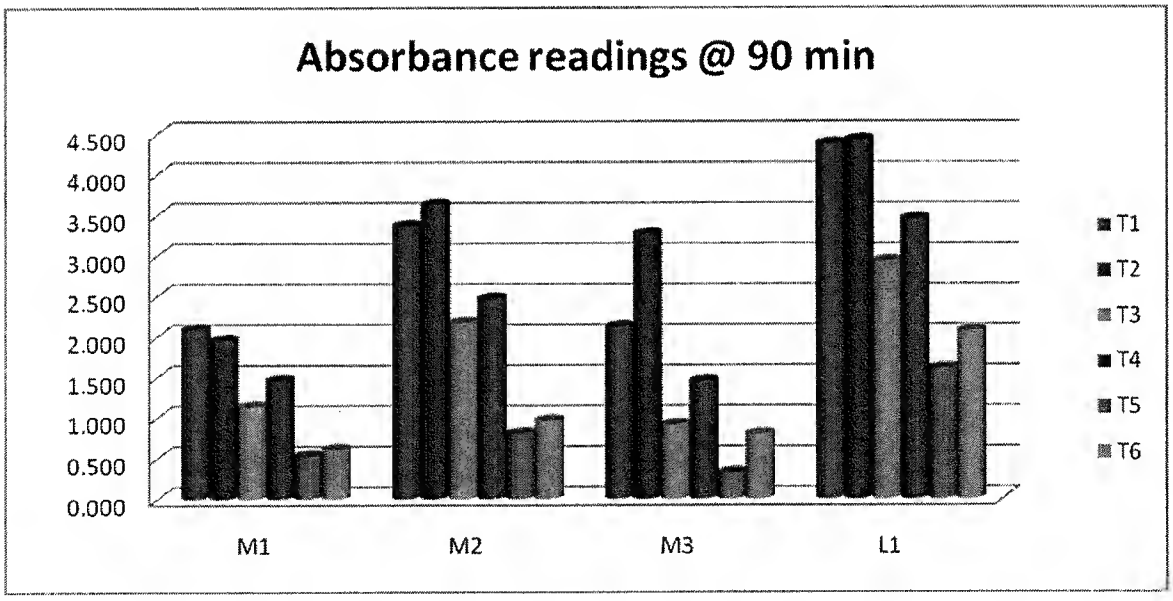


Figure 4

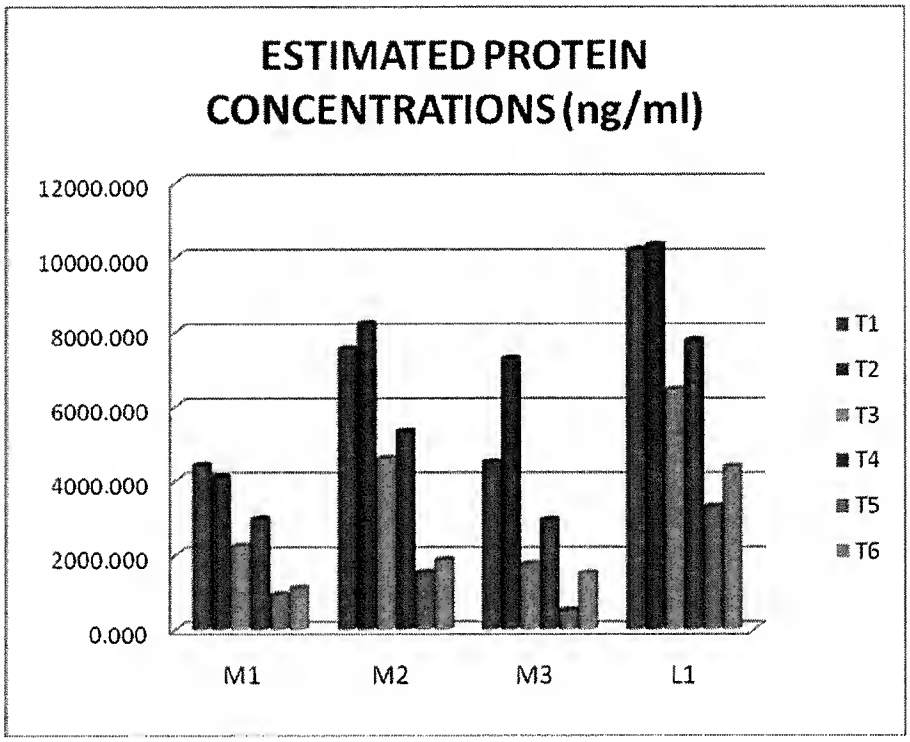
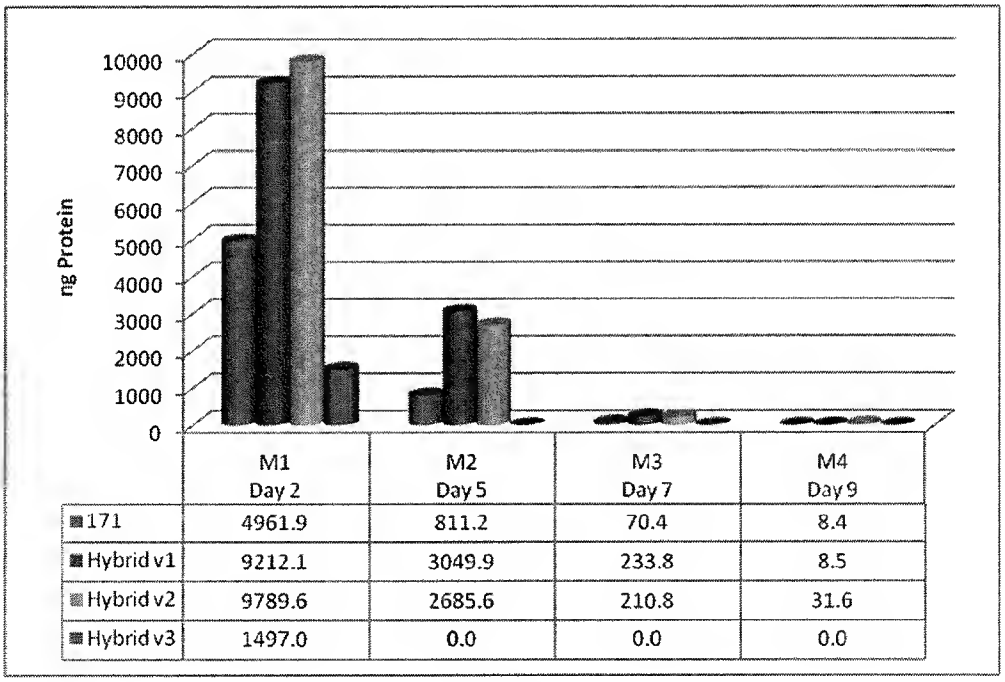


Figure 5



	M1 Day 2	M2 Day 5	M3 Day 7	M4 Day 9
171	4961.9	811.2	70.4	8.4
Hybrid v1	9212.1	3049.9	233.8	8.5
Hybrid v2	9789.6	2685.6	210.8	31.6
Hybrid v3	1497.0	0.0	0.0	0.0

Figure 6

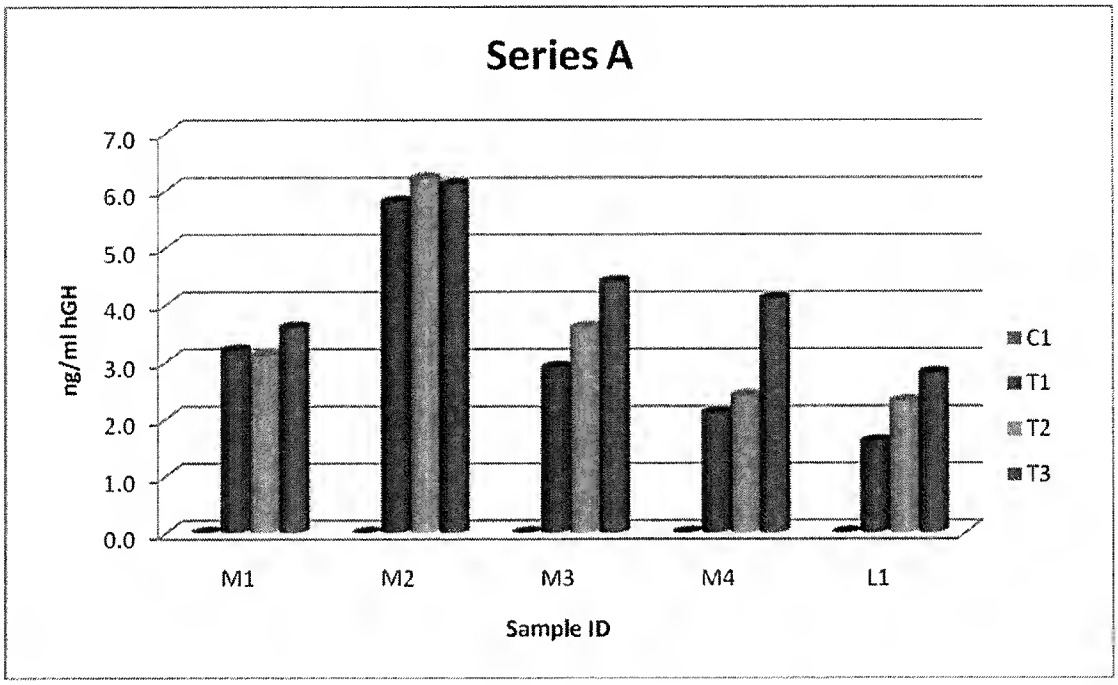


Figure 7

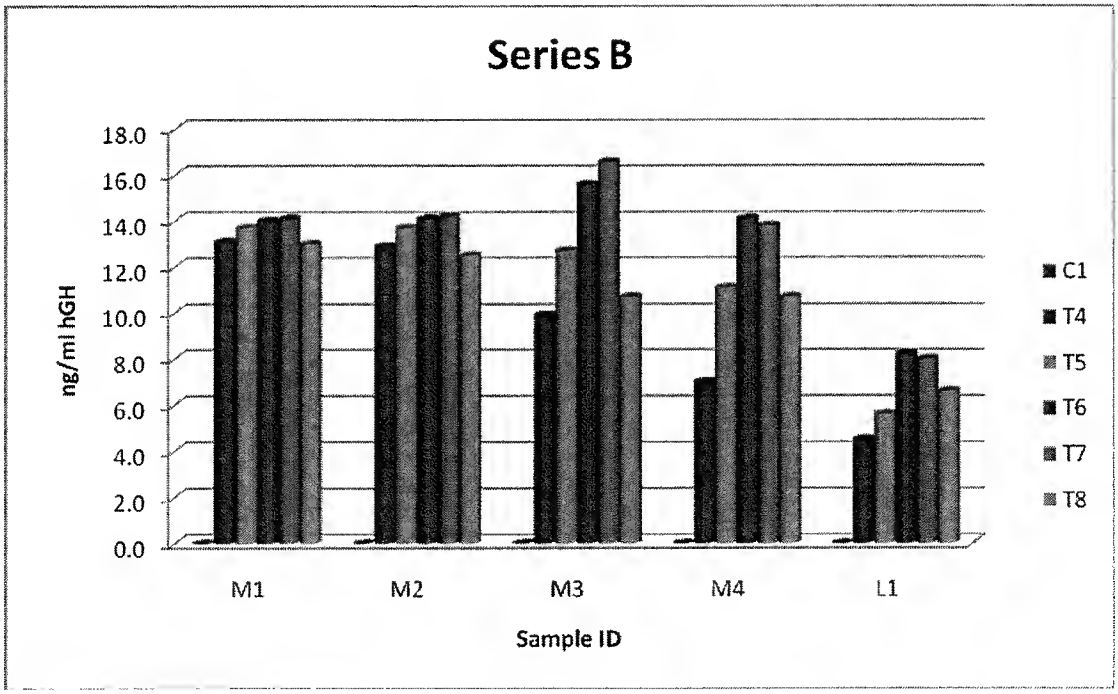


Figure 8

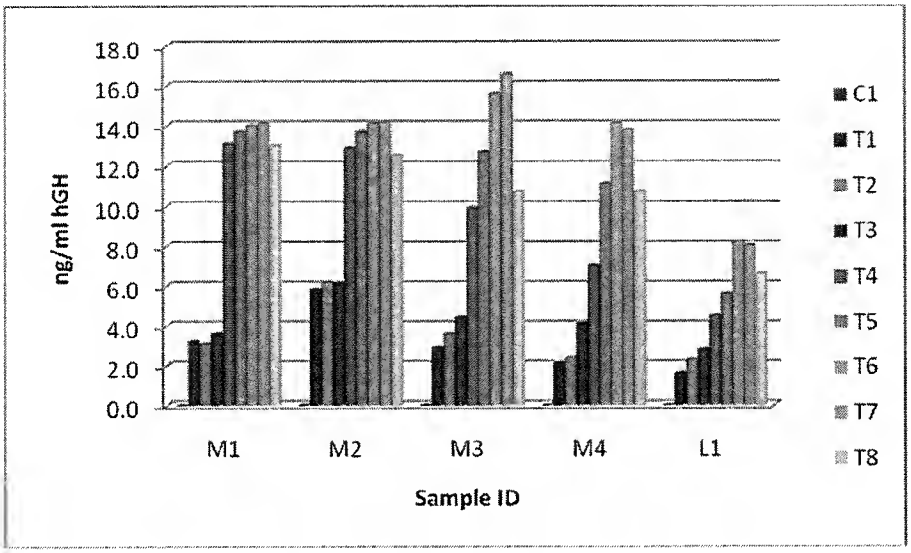


Figure 9

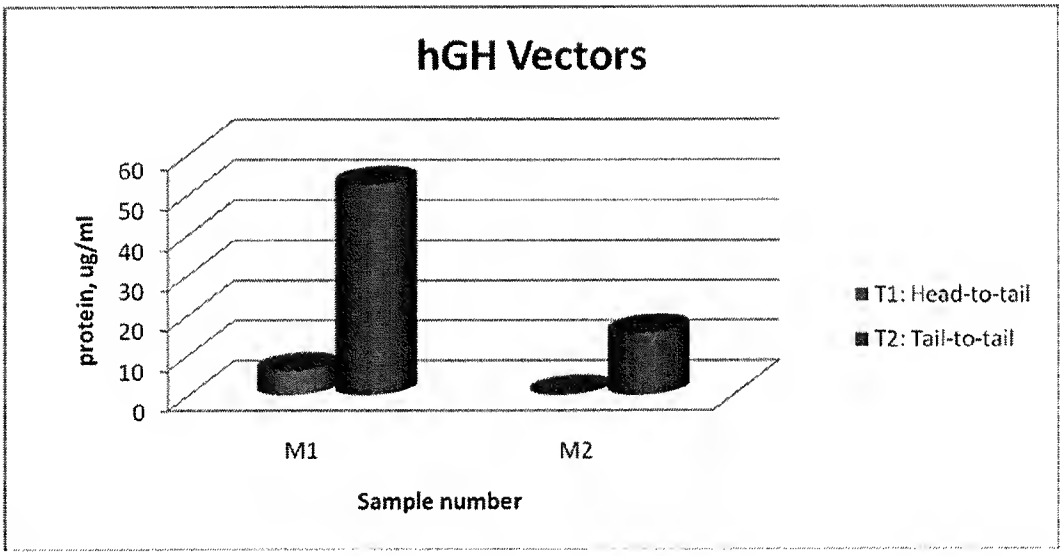


Figure 10

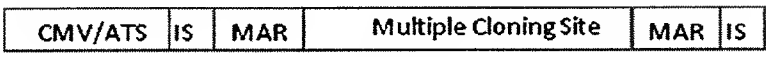


Figure 11

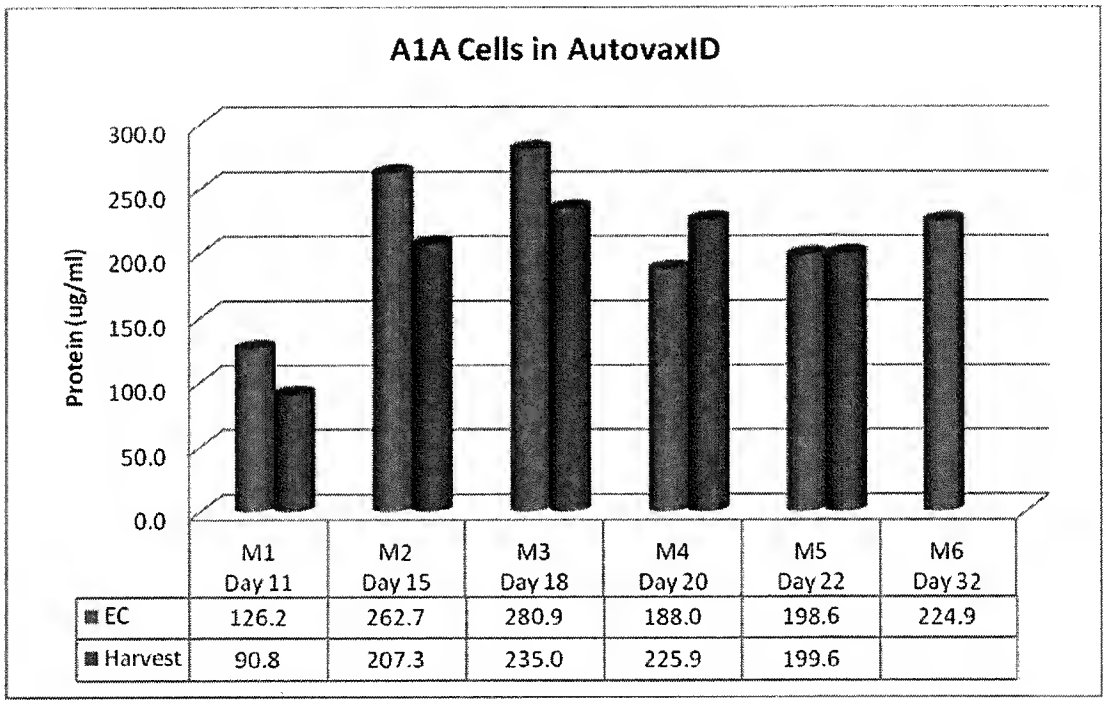

A1A Cells in AutovaxID
Protein (ug/ml)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
M1 Day 11
M2 Day 15
M3 Day 18
M4 Day 20
M5 Day 22
M6 Day 32
EC 126.2 262.7 280.9 188.0 198.6 224.9
Harvest 90.8 207.3 235.0 225.9 199.6

Figure 12